MASTER THESIS

Evaluation of the potentialities of a new β-lyase enzyme in the release of S-volatile aromatic compounds in White and Red wines

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Dissertation to obtain the degree of

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2015

Declaration of Academic Honesty

I Dor Amsalem herewith declare that the Master Thesis submitted here with the title;

Evaluation of the potentialities of a new β-lyase enzyme in the release of S-volatile aromatic compounds in white and red wines.

This thesis has been composed by myself without any inadmissible help and without the use of sources other than listed in the list of references. All person and institutions that have directly or indirectly helped me with the preparation of the thesis, have been acknowledged and that this thesis has not been submitted wholly or substantially, as an examination document at any other institution.

Lisbon,
July, 2015

Signature

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Abstract

In the past twenty years a rapid growing interest in thiols can be seen as a function of the amount of papers and research done on this topic, with the aim of understanding the biosynthesis of the main varietal thiols, as well as the desire to develop methods and ways to increase their concentration in wine. The aim of this research is to evaluate the potentialities of an additive in the form of β-lyase enzyme and the protocol used to utilize it. Two red varieties (Syrah and Touriga Nacional) and four white varieties (Alvarinho, Arinto, Encruzado and Viosinho) were used, grown and processed in Instituto Superior de Agronomia (ISA) in Lisbon, Portugal. To each varietal wine a control wine and a test wine (with enzyme addition) were made and compared for their chromatic characters and sensorial impression. Using the Statistical tools; t-Test, ANOVA, PCA and cluster analysis each treated wine was compared to its control. The results of this dissertation suggest that there are no significant differences between the treated wines and the control either in terms of chromatic characters or sensorial impressions, concluding that there may be loss of thiols to oxidation which requires a change in vinification scale.

Keywords: thiols, 3MH, 3MHA, 4MMP, β-lyase, skin-contact.

Resumo

Nos últimos vinte anos observou-se um rápido e crescente interesse em tióis, em função da quantidade de artigos e de investigação feitos sobre este tópico, com o objectivo de compreender a biossíntese dos principais tióis varietais, tal como a vontade de desenvolver métodos e modos de aumentar a sua concentração no vinho (Roland et al., 2011b). Apesar de, frequentemente, se associar os tióis varietais à casta Suvignon Blanc, muitas outras castas revelam possuir estes compostos, especialmente os 3-mercaptohexan-1-ol (3MH), acetato de 3-mercaptohexila (3MHA), 4-mercapto-4-metilpentan-2-ona (4MMP), e, de menor relevância, mercapto-4-metilpentan-2-ol (4MMPOH) (Roland et al., 2012; Coetzee & Toit, 2012), que podem contribuir positivamente para o aroma do vinho, dando-lhe odores de buxo, groselha, toranja, maracujá, entre muitos outros aromas frutados. Os tióis são produzidos pelas leveduras durante a fermentação e originam de diferentes precursores oriundos das uvas, tais como o S-3-(hexan-1-ol)-L-cisteína (Cys-3MH) ou a S-4-(4-metilpentan-2-ona)-glutationa (Glut-4MMP). Até agora, a transformação destes precursores pela acção da enzima β-liase das leveduras é muito baixa, quando comparados ao conjunto total de precursores encontrados no mosto. A conversão de Cys-3MH está apenas entre 0.1-12%, de acordo com Coetzee & Toit., (2012). Este facto torna a investigação de tióis muito mais interessante, levantando desafios de como as concentrações finais destes tióis possam ser aumentadas e este potencial inexplorado usado para influenciar o estilo de produção de vinho. Nesta tese de Mestrado, uma enzima β-liase foi adicionada ao mosto durante a fermentação, como um aditivo enológico, com o objectivo de desenvolver um protocolo para aumentar a concentração final de tióis varietais em vinhos. Foram utilizadas duas castas tintas (Syrah e Touriga Nacional) e quatro castas brancas (Alvarinho, Arinto, Encruzado e Viosinho), produzidas e processadas no Instituto Superior de Agronomia (ISA), em Lisboa, Portugal. Para cada vinho varietal foram feitos um vinho de controlo e um vinho de teste (com adição de enzima) e foram comparados nas suas características cromáticas e impressão sensorial. Utilizando ferramentas estatísticas (Teste-t, ANOVA, PCA e Clustering), cada vinho tratado foi comparado ao seu controlo. Os resultados desta dissertação sugerem que não existem diferenças significantes entre os vinhos tratados e os controlos, tanto em termos de características cromáticas como em impressões sensoriais, concluindo que pode haver uma perda de tióis por oxidação, o que requer uma alteração na escala de vinificação.

Keywords: thiols, 3MH, 3MHA, 4MMP, β-lyase, skin-contact.

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Abbreviations

AlvC: Alvarinho Control

AlvT: Alvarinho Treatment

ANOVA: Analysis of Variance

ArC: Arinto Control

ArT: Arinto Treatment

Cysgly-3-MH: 3-S-cysteinylglycinehex-an-1-ol

Cys-3MH: S-3-(hexan-1-ol)-L-cysteine.

Cys-4MMP: S-4-(4-methylpentan-2- one)-L-cysteine

EnC: Encruzado Control

EnT: Encruzado Treatment

Glut-3MH: S-3-(hexan-1-ol)-glutathione

Glut-4MMP: S-4-(4-methylpentan-2-one)-glutathione.

IBMP: 3-isobutyl- methoxypyrazine

IPMP: 3-isopropyl-2-methoxypyrazine

ISA: Instituto Superior de Agronomia

MLF – Malo-Lactic Fermentation.

PCA: Principal Component Analysis

OAV: Odor Active Values.

SBMP: 2-sec-butyl-3-methoxypyrazine

SO_2 – Sulfur dioxide

SYC: Syrah Control

SYT: Syrah Treatment

TNT: Touriga Nacional Treatment

TNC: Touriga Nacional Control

VioC: Viosinho Control

VioT: Viosinho Treatment

3MH: 3-mercaptohexan-1-ol.

3MHA: 3-mercaptohexyl acetate.

4MMP: 4-mercapto-4-methylpentan-2-one.

4MMPOH: Mercapto-4-methylpentan-2-ol.

1. Introduction

In the past twenty years a rapid growing interest in thiols can be seen as a function of the amount of papers and research done on this topic, with the aim of understanding the biosynthesis of the main varietal thiols, as well as the desire to develop methods and ways to increase their concentration in wine (Roland *et al.*, 2011b). Though the varietal thiols are often associated with Sauvignon Blanc grape variety, many other varieties have been shown to possess these compounds, especially 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA), 4-mercapto-4-methylpentan-2-one (4MMP) and to a less extent, Mercapto-4-methylpentan-2-ol (4MMPOH) (Roland *et al.*, 2012; Coetzee & Toit., 2012), all of which may contribute to the positive aromas of the wine giving it odors of Box tree, blackcurrant, grape fruit, passion fruit as well as other fruity aromas. Thiols are the product of yeasts during fermentation, and are originating from different precursors coming from the grapes, such as S-3-(hexan-1-ol)-L-cysteine (Cys-3MH), or S-4-(4-methylpentan-2-one)-glutathione (Glut-4MMP). As to now, the turnover of this precursors via the action of the yeast's β-lyase enzyme is very low comparing it to the total pool of precursors found in the must. The conversion of Cys-3MH is only between 0.1- 12% according to Coetzee & Toit., (2012). This fact makes the research of thiols much more interesting, arising challenges as to how the final concentrations of these thiols may be increased and this untapped potential used to impact wine making style. In this Master's thesis, a β-lyase enzyme was added to the wine's must during fermentation as an enological additive, with the aim to develop a protocol to increase the varietal thiols final concentration in wines. The experiment was done under enological conditions in Instituto Superior de Agronomia's (ISA) vineyards and cellar using two red varieties (Syrah and Touriga Nacional) and four white varieties (Alvarinho, Arinto, Encruzado and Viosinho), with the collaboration of AEB group. The wines were compared in their sensorial impressions on the 25/06/2015 by a panel of 10 trained and experienced tasters with the focus on tropical notes as interest attribute and the results of the tasting is presented and analyzed here, together with a chromatic comparison of each of the wines.

2. Literature Review

2.1 Wine's Aromas

Wine is a very complex matrix in terms of aroma compounds, having more than 1000 volatile compounds that have been identified so far. (Roland *et al*., 2012). It is important to note that in this thesis research I will focus on compounds that are "varietal aromas", not implying that these aromas or volatile compounds, may exist in one variety and not in another, but that each grape variety will have a different aroma profile thanks to a particular combination of the compounds, which means that these volatile compounds or their precursors can be found in the musts and wines produced from several different grape varieties. (González-Barreiro *et al*., 2015).

2.1.1 Classification of aromas

The aromatic compounds of wine can be classified according to the time when they were formed, as was proposed by Drawert (1974) (Roland *et al*., 2011b, Roland *et al.,* 2012) resulting in four classes of compounds: ***Varietal aroma compounds*****;** these compounds may be the result of odorless precursors that can be found in grapes (Roland *et al.,* 2012).More commonly found in a bound form, linked to a non-volatile group (amino acid, sugar, etc.) by a covalent bond but can also be in a free, volatile form. The first form is considered to be a direct and specific precursor, that can be the product of the wine making process, and that the original structure of the precursor is preserved to an extent in the new aromatic compound, this comparing with most of yeast's substrates that go through a complex series of bio-chemical reactions, in which the primary structure of the precursor is not recognizable in the resulting aromatic product, being a different class of aromatic compounds (Roland *et al*., 2011b).

Pre-fermentation aromas; occur between harvest and alcoholic fermentation, during the crashing of the berries and an enzymatic reactions. (Roland *et al*., 2011b)

Fermentation aromas; refers to the secondary products of the metabolism of micro-organism of the fermentation process, i.e. yeast, lactic acid bacteria, resulting in an aromatic profile of vinous and fruity.

Post-fermentation aroma; via chemical and bio-chemical reactions that modify volatile compounds, occurring in the process of wine aging and may attribute to the complexity of old wines (Roland *et al*., 2011b).

2.1.2 Chemical classes of varietal compounds in wines

Looking at the grape berry growth and development, it is possible to notice two sigmoidal growth periods separated by a lag phase; berry formation and berry ripening, respectively (Coombe & McCarthy*.,* 2000). At the second stage of the development we may see the formation and increase in second metabolites, such as volatile flavor compounds. The aroma compounds, or their precursors, are found in the flash, and more considerably in the skin of the berry (González-Barreiro *et al.,* 2015).

The next text will be focus on a few of the main varietal volatiles in grapes.

2.1.2.1 Terpenes

The family of terpenes compounds are greatly common around the plant kingdom, approximate to be around 4000 in number. (Ribéreau-Gayon *et al.*, 2006). Monoterpenes (10 carbon atoms) and sesquiterpenes (15 carbon atom), are likely to be odoriferous, with the first one varying in forms: simple hydrocarbons (limonene, myrcene, etc.), aldehydes (linalal, geranial, etc.), alcohols (linalol, geraniol, etc; some of these alcohol compounds are part of the most odoriferous), acids (linalic and geranic acid, etc.) and even esters (linalyl acetate, etc.). According to (González-Barreiro *et al.,*2015) terpenes can be found in most grapes, contributing to the fruity (citric) and floral aromas of the wine, though some of the about 40 identified terpenes in grapes may have resin aromas. Terpenes have a quite low perception threshold concentrations, ranging from tens of µg/l (such as Linalol; 50 µg/l) to as little as few hundred µg/l (as Nerol; 400 µg/l) (Ribéreau -Gayon *et al.,* 2006). Muscat's wine and grapes are characterized by this family of compounds, showing high concentrations often increasing those of the perception threshold. (Ribéreau-Gayon *et al.,* 2006). The important once being linalool, geraniol, nerol and citronellol. (Styger *et al.,* 2011). Some of these compounds may be found in a non-volatile state at the grapes and must; having a sugar group attached to it, i.e. glycosylated form, requiring the liberation of the volatile terpene group. The bound form is more abundant in the grapes than free aromas, though the concentration of both is higher in the skin of the grape than in the flesh, and the proportion of the two forms is grape variety depended. (Ribéreau-Gayon *et al.,* 2006, Styger *et al.,* 2011). Its localization in the skin resulted in increase of both bound and free forms of monoterpenes after skin contact treatment. (Styger *et al.,* 2011).

2.1.2.2 C13-Norisoprenoids Derivatives

As applied from their name; these compounds consist of 13 atoms of carbon, resulting from an oxidative degradation of carotenoids (40 carbon atom terpenes). (Ribéreau-Gayon *et al.,* 2006). Chemically wise, this group of compounds can be divided into two forms; *Megastigmane*, which includes β- damascenone, β-ionone, 3-oxo-α-ionol, β-damascone, and 3-hydroxy- β-damascone, and

Non-megastigmane, with a few important compounds from this group, such as TDN (1,1,6-trimethyl-1,2-dihydronaphthalene), with a distinctive kerosene-like odor; TPB ((E)-1-(2,3,6-trimethylphenyl)buta-1,3-diene), may contribute as strong cut-grass aroma in white wines, actinidiols and vitispirane, which have odors reminiscent of camphor. (González-Barreiro *et al.*, 2015). Many of the Norisoprenoids are in their nonvolatile glycosides form in the grape, and are being hydrolyzed (acid or enzymatic) during fermentation and storage to release the aromatic compound. Even though their concentration in wine in very low, the perception threshold for most of them is also very low, which makes them an important contributor to wine's aroma. (González-Barreiro *et al.*, 2015). Like most varietal aromas, also Norisoprenoids are highly influenced by the yeast strain used. (Styger *et al.,* 2011).

2.1.2.3 Methoxypyrazines

Nitrogenated heterocyclic products originating from the metabolism of amino acids from the grapes. These compounds may contribute to the vegetal profile of the wine via molecules such as 3-isobutyl-methoxypyrazine (IBMP), 2-sec-butyl-3-methoxypyrazine (SBMP), and 3-isopropyl-2-methoxypyrazine (IPMP); bell peppers or green gooseberries, asparagus or green bean pea or bell pepper respectively. (González-Barreiro *et al.*, 2015). Maximum levels of pyrazines can be found in veraison with a decrease of it during ripening. The final consternations are affected by the origin and climate; high temperature and light exposure leads to degradation. Considering that, canopy management could influence their levels. At harvest, the localization of pyrazines is mostly in the skin, and therefore influenced by skin contact time. (Coetzee & Toit*.,* 2012).

2.1.2.4 Sulfur-containing compounds

When classifying these compounds, we may consider two groups; one group contains volatile Sulphur compounds with negative aromas, such as the formation of H_2S by wine yeast, resulting in rotten egg aroma, or the occurrence of other Sulphur containing compounds, as thioacetic acid esters and mercaptans, arising because of low redox potential and might cause aromas such as cooked vegetables, onion and cabbage. (Coetzee & Toit*.,* 2012). On the contrary, a second group may attribute to the positive aromas of the wine through the occurrence of volatile or varietal thiols. (Coetzee & Toit*.,* 2012). The same compounds, either positive or negative, may change their impact on the wine, depending on their absolute and relative concentration in the wine. (Pretorius *et al.,* 2007). Five groups of sulfur compounds may be considered; (1) hydrogen sulfide (rotten egg), (2) methanethiol (methylmercaptan - cooked cabbage), (3) dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide (cabbage, cauliflower, and garlic), (4) methylthioesters (S-methyl thioacetate, S-methyl thiopropanoate, and S-methyl thiobutanoate - cooked cauliflower, cheesy, and chives aromas) (5)

volatile thiols (passionfruit, grapefruit, gooseberry, guava, and box hedge aromas) (Pretorius *et al.,* 2007).

2.2 Varietal Thiols

The volatile thiol compounds are not in their free from in the berry or must, but are linked to a functional group, such as alcohols, esters and ketones. (Coetzee & Toit*.,* 2012). Looking at the main varietal thiols, we may consider; 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and its acetate, 3-mercaptohexyl acetate (3MHA). Each compound may contribute to the aromatic profile of the wine, with aromas such as Black currant, box tree, grape fruit and passion fruit. (Tominaga *et al.,* 1996; Tominaga *et al.,* 1998a; Roland *et al.,* 2012 Coetzee & Toit*.,* 2012; Masneuf-Pomarède *et al.,* 2006). A firth compound, 4-mercapto-4-methylpentan-2-ol (4MMPOH), with less importance than the first three; having a concentration that is rarely above its olfactory threshold (Coetzee & Toit*.,* 2012), may attribute to positive wine aromas. These compounds are usually associated with Sauvignon Blanc grapes, as it was first published by Du Plessis and Augustyn (1981) that South African Sauvignon Blanc presents a guava like aromas that are mostly due to 4MMP (Roland *et al.*, 2011b). This grape variety had become very popular cultivater, with perception of tropical aromas, highly contributed by the volatile thilols. The disscovery of the compunds in grapes with the increasing popularity of this variety have statred an influx of reaserch foucisng primarly on Sauvignon Blanc gapres. (Roland *et al.*, 2011b, Roland *et al.*, 2012, Coetzee & Toit*.,* 2012).

2.2.1 Precursors and Location in the grapes

Early trials done by Darriet *et al.,* (1995) had shown, in wine made from Sauvignon Blanc grapes, the presence of a precursor that through the work of an enzyme, during the fermentation process, is releasing thiols. That research and others (Tominaga *et al.,* 1998a, Peyrot des Gachons *et al.,* 2002) allowed to identify the precursor as an amino acid with a thio-ether bond that was named cysteinylated S-conjugate precursors and glutathione conjugates (Peyrot des Gachons *et al*., 2002, Roland *et al.*, 2011a, Roland *et al.,* 2012).

The main precursors addressed in literature are:

S-3-(hexan-1-ol)-L-cysteine (Cys-3MH).
S-4-(4-methylpentan-2- one)-L-cysteine (Cys-4MMP).
S-3-(hexan-1-ol)-glutathione (Glut-3MH).
S-4-(4-methylpentan-2-one)-glutathione (Glut-4MMP).
(Figures 1 and 2 present the chemical structure of each precursor).

A B

Figure 1: Chemical structures of Cys-3MH (A) and Glut-3MH (B)

Figure 2: Chemical structures of Cys-4MMP (A) and Glut-4MMP (B)

The volatile thiols are almost absent in the grape, though according to (Capone *et al.,* 2010), a concentration of about 100 ng/l of 3MH was seen at harvest time. The final thiol's concentration in wine is depended also on the pool of precursors available in the must, thus it is important to know the location of the precursors to be able to fully take into advantage their potential in the wine. (Peyrot des Gachons *et al*, 2002). At harvest Cys-3MH was more abundant in the skin than in the pulp in Sauvignon Blanc grapes (Peyrot des Gachons *et al.,* 2002, Allen *et al,* 2011.). Cys-4MMP was mostly in the flesh (about 80%). To be precise, the concentration of the precursor Cys-3MH in the skin was shown to be eight times than in the flesh. This statement was supported using the results of skin contact trials; showing an increase in precursors in the juice with 15% increase for Cys-4MMP, 30% increase for 4MMPOH precursor and 50% increase in Cys-3MH, after 19 hours of skin contact in laboratory conditions. The precursors of 4MMP and 4MMPOH can mostly be found in the pulp of the grape. (Peyrot des Gachons *et al.,* 2002). Glut-3MH has been found to be up to 35 times higher than that of Cys-3MH and it thus seems that the cysteine conjugate is quantitatively not the main precursor (Capone *et al.,* 2010). However, the conversion rate from the precursor to the free thiol has been found to be more efficient for the cysteinylated precursor compared to the glutathionylated precursor in model juice (Winter *et al.*, 2011, Coetzee & Toit*.,* 2012). According to Roland *et al.*, (2011a), Glut-3MH

is equally distributed between skin and pulp, and its levels are depended on glutathione content in the vine. Same dependency can be observed for Glut-4MMP. (Makhotkina *et al.,* 2014). Research done on Sauvignon Blanc and Melon B. grapes, comparing the precursors distribution according to grape variety, had shown differences among the two varieties, with higher occurrence of precursors for Sauvignon Blanc, but it is important to note the observation that considerable amounts of all precursors were located in the skin, regardless of the variety. (Roland *et al.,* 2011a).

2.2.2 Biogenesis and occurrence of precursors and volatile thiols

The biogenesis of the varietal thiols have been the research of a lot of papers. The precursors already mentioned are synthetized in the grape, released to the must and are going through a series of reactions before releasing the volatile compound. (Figure 4 present the chemical structure of the main thiols). (Roland *et al.,* 2011b, Roland *et al.,* 2012, Coetzee & Toit*.,* 2012). As described in their review, (Roland *et al.,* 2011b) the biogenesis of 4MMP and 3MH can be commonly explained by three pathways; cysteinylated precursor, glutathionylated precursor and C_6 unsaturated compounds. The pathways suggested are still being researched and the exact mechanisms is not yet fully understood (Coetzee & Toit*.,* 2012), though the literature presents information regarding different parts of the different mechanisms. The first pathway; Cys-3MH is a direct precursor of 3MH (Tominaga *et al.,* 1998b). Cys-3MH is structurally similar to cysteine, hence its uptake by the yeast is thought to happen via amino-acid transporters. (Coetzee & Toit*.,* 2012). The precursor is processed by β-lyase enzyme originating from the yeast, especially for Cys-3MH, being more abundant in the grapes than Cys-4MMP. (Roland *et al.,* 2011b). The enzyme works and cleaves the Carbon-Sulphur linkage (Dubourdieu *et al.,* 2006, Coetzee & Toit*.,* 2012). That was evidence when the gene encoding for that enzyme was deleted from the yeast and as a result 4MMP levels were reduced. (Howell *et al.,* 2005). The second pathway is the glutathionylated pathway. Research done on this pathway using Glut-3MH, had presented different results, either rendering it as pro-precursor of Cys-3MH or precursor of 3MH (Roland et *al.,* 2010, Thibon *et al.,* 2011, Roland *et al.,* 2011b). When considering it to be a pro-precursor to Cys-3MH, a suggested pathway involves the elimination of toxic compounds in the grape as a response to biotic or abiotic stress, when it is conjugated with glutathione by S-glutathione transferas. From the resulting product a glutamic acid is removed by γ-glutamyltrans-peptidase and glycine by cardoxypeptidase. These reactions will result in the Cys-3MH product. (Coetzee & Toit*.,* 2012). The last pathway is through a sulfur addition (i.e. H_2S or glutathione) to a C_6 unsaturated compounds, such as (E)-2-hexenal (Figure 3), at alcoholic fermentation. As reported by Capone *et.al,* (2010), Allen *et al.,* (2011) and Harsch *et al.,* (2013) Glut-3MH can be a product of enzymatic or chemical conjugation of glutathione and (E)-2-hexenal. An intermediate to that conjunction, is suggested in the form of aldehyde that also seems to be an additional potential precursor of 3MH.

(Capone & Jeffery*.,* 2011a). In the must, (E)-2-hexenal forms from unsaturated lipids, in the present of oxygen. (Subileau *et al.,* 2008, Harsch *et al.,* 2013). In the work of Roland *et al.,* (2011a) it was shown an increase 3MHA and 3MH production with glutathione or hexenal additions. Conversion of (E)-2-hexen-1-ol to (E)-2-hexenal is favorable by yeast in the presence of oxygen. (Harsch *et al.,* 2013). The research done in attempt to understand the different pathways is an important one and have been shown progress over the years. That being said there is much work to be done in order to paint a complete picture that will account for the vast gaps between the concentration of precursors and that of volatile thiols in the wine. (Coetzee & Toit*.,* 2012). For example, according to (Roland *et al.,* 2012), 90% of the 3MH can't be explained by the three pathways suggested here, and only 20% of 4MMP, in Sauvignon Blanc was the result of Glut-4MMP. To account for the evolution of the processors, more intermediate forms may be identified, as in the research of (Capone & Jeffery., 2011b), where a new conjugate, 3-S-cysteinylglycinehex-an-1-ol (Cysgly-3-MH) was identified. This dipeptide seems to be an intermediate of cysteine and glutathione on the pathway of 3MH biogenesis.

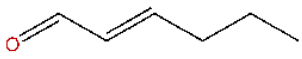
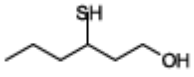
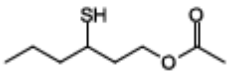


Figure 3: Chemical structure of (E)-2-hexenal.

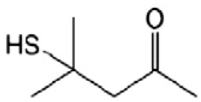
As for 3MHA; its biogenesis is by the action of the yeast ester-forming alcohol acetyltransferase, from 3MH and acetyl-CoA, which is encoded by the ATF1 and ATF2 genes, and is differ in its ability to catalyze the reaction between yeast strains (Swiegers *et al.,* 2009, Coetzee & Toit*.,* 2012, Villas-Boas *et al.,* 2014). The occurrence of 3MHA is usually up to 10% of 3MH concentration. (Dubourdieu *et al.,* 2006).



3MH



3MHA



4MMP

Figure 4: the chemical structure of the 3 main thiols: 3MH, 3MHA and 4MMP.

When talking about occurrence and concentration (table 1) levels of thiols, the scale is a small one, with 4MMP recorded at the range of 4-40 ng/l in wines (Coetzee & Toit., 2012) with a perception threshold of less than 1 ng/l (Tominaga *et al.,* 2000). 3MH present perception threshold of about 60 ng/l, while 3MHA is around 4 ng/l, both in hydro-alcoholic solutions (Tominaga *et al.*, 2000, Coetzee & Toit*.,* 2012), and with a vast occurrence in wines, occurring at up to 18,000 ng/l for 3MH and 2500 ng/l for 3MHA (Roland *et al.,* 2012; Coetzee & Toit*.,* 2012). 4MMPOH shows a concentration that is rarely above its olfactory threshold of 55ng/l. (Coetzee & Toit*.,* 2012). The low perception threshold of 3MHA makes it a significant contributor to wine, and its occurrence in wine is with its two enantiomeric forms, R being four times higher in perception threshold than the S form and presenting aroma of passion fruit. The S form is herbaceous and is found three times higher in concentration comparing with R. (Tominaga *et al.,* 2006). 3MH is also found in its two enantiomeric forms, usually presenting an equal amount of both R and S forms in white dry wines, but with different perception thresholds and possible aroma; The R form reminiscent of grape fruit and citrus peel with perception threshold of 50 ng/l of and the S form has a 60 ng/l threshold and attribute to the passion fruit aroma of the wine. (Tominaga *et al.,* 2006). At too high concentrations these compounds might damage the wine's aromatic profile and cause sweaty or cat urine impression, at the levels pf mg/L. (Swiegers *et al.,* 2009; Coetzee & Toit*.,* 2012; Peña-Gallego *et al.,* 2012).

Compound (Abbreviation)	Odors	Perception threshold in model solution (ng/L)	Concentration in Literature (ng/L)	References
4MMP	Box tree, Passion fruit, Bud.	0.8	Until 400	a, b, d
3MHA	Box tree, Grape fruit	4.2 in racemic mixture	Until 2500	a, b, c, d
3MH	Grape fruit, Passion fruit	60 in racemic mixture	Until 19000	a, b, d
4MMPOH	Citrus zest	55	Until 90	a

Table 1: Main volatile thiols in wines, their occurrence and concentration

a – Roland et al., (2011b); b - Coetzee & Toit*.,* (2012); c - Tominaga *et al.,* (1996); d- Tominaga *et al.,* (1998b)**.**

2.2.3 Viticulture Practices

Research done on cysteine precursors, in Sauvignons Blanc grapes from Bordeaux for two years where the level of Cys-4MMP, Cys-4MMPOH and Cys-3MH were checked one month before harvest had shown vast differences in concentration; ripening had a direct effect on processors level by increasing it. (Roland *et al.,* 2011b). Cys-3MH development in grapes may be increased through water deficit (Chone *et al.,* 2006), and its amount is directly proportional to it. Cys-4MMP is inversely proportional to the water deficit. (Roland *et al.,* 2011b). Temperature shocks (hot and cold), UV-C radiation, biochemical stimulation or botrytis bunches (Allen *et al.,* 2011; Thibon *et al.,* 2011) may also increase Cys-3MH levels. Both Cys-3MH and Glut-3MH may be influenced by the soil composition, environmental conditions and the elevation, as it seen in Koshu grapes. In the lowest elevation, the most rapid accumulation was observed. (Kobayashi *et al.,* 2010). The vineyard management has an impact on the must composition, which is appear to be linked to the precursor's concentration. Together with the water deficit impact, also the vine nitrogen status could influence the levels of all cysteinylated precursors; low nitrogen can decrease their concentration. (Chone *et al.,* 2006, Roland *et al.,* 2011b). Sulfur additions with sufficient nitrogen supply may increase the concentration of these precursors. (Roland *et al,* 2011b, Coetzee & Toit*.,* 2012). Glutathione precursors may be influenced by similar environmental conditions that might limit glutathione content in the vine, thus resulting in lower glutathione precursors (Chone *et al.*, 2006, Roland *et al.,* 2011a).

2.2.4 Enology Practices

When considering the different enology practices and their impact on volatile thiols levels, we may follow the line of production and processing of the grapes to evaluate the different factors; **Harvest and transport;** according to a study done by (Capone & Jeffery., 2011a), when machine harvested grapes, that were transported over 800 km for about 12 hours were compared with hand-picked grapes with no transportation, the first showed Cys-3MH concentration 10 times higher than the last, while Glut-3MH did not present the same increase. Considering the fact that Cys-3MH appear to be easily utilized, this is an important observation (Coetzee & Toit*.,* 2012); that seems to be caused by more grape damage and longer skin contact time (Capone & Jeffery., 2011a). The additions of antioxidant agents, when transporting grapes, with ascorbic acid alone or with 50 mg/L SO_2 did not affect much the concentration of Cys-3MH. When no antioxidant was added an increase of Cys-3MH was observed, though also phenolic oxidation was visible. In terms of Glut-3MH, ascorbic acid with moderate levels of SO_2 exhibit an increase in the precursor. The use of 500 mg/L SO_2 in combination with ascorbic acid had resulted in decrease in both precursors. That could be explained due to the SO_2

impact as an inhibitor to some enzymes, it also could be in the conversion stage of Glut-3MH to Cys-3MH, and possibly also at the construction of (E)-2-hexenal, by preventing necessary oxidation of fatty acid or inhibit enzyme in the chain of reactions. (Coetzee & Toit*.,* 2012). This was confirmed by a study done by Capone & Jeffery., (2011a) who found that most of the Cys-3MH identified in the juice is already present in the berry, whereas most of the Glut-3MH is formed in the juice during processing. (Kalua & Boss*.,* 2010). Finally, it seems that normal levels of SO_2, addition of O_2 to the must, with enough time for enzymatic reactions, can result in increase of precursors of 3MH. (Roland *et al.,* 2010b, Coetzee & Toit*.,* 2012, Coetzee & Toit*.,* 2012). **Pre-fermentation treatments;** as already mention, in trials done by (Peyrot des Gachons *et al.,* 2002) with skin contact of Sauvignon Blanc grapes, an increase in thiols precursors in the juice was observed, showing a favorable results for Cys3-MH, with little effect on Cys-4MMPOH and Cys-4MMP. In the same experiment, an increase in the temperature during the skin contact had resulted in a considerable increase in Cys-3MH in the must, but allowed only a slight increase in 4MMPOH and 4MMP precursors. Due to slow diffusion time of the precursors to the juice, long periods of time gave higher extraction results. (Peyrot des Gachons *et al.,* 2002, Coetzee & Toit., 2012). Higher pressed juice had showed higher level of 3MH precursors (Pate *et al.,* 2010), though due to an increase in phenolic compounds and the oxidation process, the wines presented a decrease of 50% in 3MH and 3MHA. Therefor it is advised to protect and process these pressed juice to prevent to loss of thiols. (Roland *et al.,* 2011b). Cold-soaking of a seven period day had not changed much the precursor's concentration, though a significant increase in 3MH and 3MHA was visible (up to 55%) in the corresponding wines. (Coetzee & Toit*.,* 2012). **Fermentation;** the process of fermentation is greatly impacted by many factors, such as the composition of the must, the clarification process, the yeast strain used, the temperature; these factors and more will influence the final aromatic profile of the wine. (Masneuf-Pomarède *et al.,* 2006, Swiegers *et al.,* 2009)**.** When the temperature is considered; low values (10-15 °C) are in interest for they may enhance the production of a number of positive volatile compounds (in the form of esters, acetates, and medium chain- fatty acids), nevertheless it might also result with a stuck fermentation. Studies done on Sauvignon Blanc wines, in which the aromatic characteristic is due to volatile thiols, had shown that the final concentrations of the three main volatile thiols (4MMP, 3MH and 3MHA) were higher when the temperature during fermentation was higher (20 °C compare to 13 °C) and that was shown to be regardless of the yeast strain used. (Masneuf-Pomarède *et al.,* 2006). That doesn't say that the choice of yeast doesn't impact the final concentrations as well; the ability to release these thiols vary with the strain of yeast, for example *S. bayanus var. uvarum* strains and hybrids *S. cerevisiae×S. bayanus var.* have shown to possess high abilities to the task; in the same paper, (Masneuf-Pomarède *et al.,* 2006) had tested the influence of different yeast strains (three S. *cerevisiae* and one hybrid strains S.*cerevisiae×S. bayanus var. uvarum*) on the production of the three main volatile thiols; their results

suggested significant differences between the strains in terms of thiols release abilities. According to (Swiegers *et al.,* 2009), when seven yeast strains were tested they presented significant differences in the volatile thiols, though the basic chemical composition showed minor differences. In addition, a large degree of correlation between the flavors compound analysis and the sensory analysis. The use of different yeast strains lead to variations in the major thiols compounds (4MMP, 3MH and 3MHA), and a unique volatile flavor profile. (Swiegers *et al.,* 2009). Not just the use of different yeast strains may result in changes of thiol concentration, but also co-inoculation of two S. cerevisiae may modify this characteristic. (King *et al.,* 2008) this was further supported by (King *et al.,* 2010), showing not just differences in compounds composition as result of different yeast treatment, but also a large variances in consumer acceptance. Additionally, in their research (Anfang *et al.,* 2009) presented results suggesting possible interactions between some S. *cerevisiae* and certain non-Saccharomyces yeasts, which have the potential to increase varietal thiols (specially 3MHA) in Sauvignon Blanc wines. It seems as if no matter what the yeast strains used, there is a limitation to its ability to process precursors into volatile thiols, as already mention. The conversion of Cys-3MH into 3MH and 3MHA was between 0.1 to 12% and that of Glut-3MH was less than 5% in different researches that were conducted. (Coetzee & Toit*.,* 2012). In terms of finning off the wine; already in the process of thiols identification, it was noticed that addition of copper can remove these tropical characteristics, due to reaction between the copper and the sulfur compound resulting with insoluble odorless sulfides (Swiegers *et al.,* 2009). **At and after bottling;** volatile thiols might be affected from the oxygen entering the wine and the quality of the closure. (Coetzee & Toit*.,* 2012). If O_2 filtrates to the wine through the closure, the wine might have a significantly lower levels of SO_2, ascorbic acid and also volatile thiols, as it was found in Sauvignon Blanc grapes. (Lopes *et al.,* 2009; Ugliano *et al.,* 2011). The oxidative loss of thiols (see also section 2.2.5) may be prevented by additions of glutathione, 20 mg/L just before bottling as it was shown in (Ugliano *et al.,* 2011) that 3MH levels were increased 6 months after bottling in these conditions.

2.2.5 Oxidative loss of thiols and Negative sulfur compounds

In their precursor form, thiols are quite stable against oxidations thanks to C-S bond (Roland *et al.,* 2010b). Volatile thiols, together with other wine compounds, are nucleophilic compounds, likely to react with quinones; a reactive electrophilic oxidation intermediates in wines. The reaction of both may result in the loss of varietal thiols. (Waterhouse *et al.,* 2012). In their research, Waterhouse *et al.,* (2012) tested the competitive kinetics of nucleophilic compounds with quinones. The results suggest that the anti-oxidant compounds (SO_2, glutathione and ascorbic acid) and H_2S will react favorably with quinones than volatile thiols would. That may result in the perseveration of these aromatic compounds if the wine is undergoing oxidation. Three mechanisms seems to govern the oxidation of these thiols;

(1) O_2 and iron might form H_2S, (2) reaction with electrophilic compounds or (3) chemical reactions with products such as quinone, i.e. products of phenolic oxidation. (Coetzee & Toit*.,* 2012). The Hydrogen sulfide, H_2S is a product of the sulfate reduction sequence (SRS) pathway that occurs inside the yeast's cell. As part of the bio-synthesis of required organic sulfur compounds during fermentation (such as Cysteine and Methionine). HS^- ions are used together with compounds that are derived from nitrogen metabolism (*O*-acetyl serine and *O*-acetyl homoserine) for the synthesis process. However when nitrogen supply in insufficient, the free H_2S that is derived from the HS^- is accumulated in the cell and then released to the must. (Pretorius *et al.,* 2007) H_2S is highly reactive and easily combined with other compounds in the wine, might resulting in other negative aromatic compounds (Vermeulen *et al.*, 2005). To prevent or decrease the formation of H_2S a sufficient nitrogen conditions are required and the attention to other environmental conditions is highly recommended. (Pretorius *et al.,* 2007)

2.3 β-lyase Enzyme

The use of enzymes in flavor-chemistry to biocatalyst different reactions have been well-known methodology. (Wakabayashi *et al.,* 2002). The β-lyase enzyme was established to be involved in the release of 4MMP as well as 3MH and it's encoded by the gene IRC7 in S.*cerevisiae* yeast. (Villas-Boas *et al.,* 2014). Concerning Cys-3MH; the precursor is taken up by the yeast during fermentation via transporters such as Gap1p. The Glut-3MH is transported via Opt1p. In the cytoplasm of the yeast the precursors are transformed by the β-lyase enzyme. (Marullo et al., 2013). Different proteins had been reported to have the ability to cleave synthetic or natural cysteinylated precursors of 3MH, however with different efficiencies. This may be a function of the kind of precursor, its concentration and most likely other environmental parameters in the must. (Marullo *et al.,* 2013). In terms of 4MMP, it seems as if the Irc7p enzyme is the unique β-lyase that transforms its precursors. (Marullo *et al.,* 2013). According to Gardner *et al.* (2013) it was only the knockout of IRC7 gene the lead to a significant decrease in both 4MMP and 3MH (about 96 and 40% respectively). The bioconversion of the precursors into their volatile form had been shown to be affected by Nitrogen Catabolite Repression (NCR), i.e. genes that encodes for transcriptional regulators with the ability to impact other gene's full expression under rich nitrogen source conditions. (Marullo *et al.,* 2013). Considering that, the manipulation of NCR genes, either genetically or by the use of urea as a nitrogen source had shown to result in an increase in thiol synthesis. (Gardner *et al.,* 2013).

2.4 Varieties

A well-known variety for its varietal thiols is Sauvignon Blanc. With wines produced from this variety being increasingly popular, also since its range of styles can be influenced both in the vineyard and the cellar. (Coetzee & Toit*.,* 2012). Varietal thiols are not unique to this cultivator and can be found in many

other varieties; Riesling, Colombard, Semillon, Cabernet Sauvignon and more (Roland *et al.,* 2011b; Coetzee & Toit*.,* 2012). A primary reseacrh, studing the presence of thiols in white Portugues garpe varities was done by Ferreira *et al.*, (2011), starting a growing intersest in the topic, showing the presence of volatile thiols, comparing it to that of Sauvingon Blanc wines, as will be described here.

2.4.1 Red Varieties

2.4.1.1 Syrah

The grape variety Syrah is originated from France and is capable of producing wines with strong colors, high intensity and violet shades in the duration of its youth. Aromatically, it is nonaromatic grape (Martínez-Gil *et al.,* 2012), but during its vinification and as wine it may present a complex aromatic profile, with floral, fruity, spices and animals notes. The richness in tannins and its strength give the Syrah made wines well ageing potential. (Chaves, 2012).

2.4.1.2 Touriga Nacional

This grape variety originated from the north of Portugal, though it's grown in all the country's regions. It is suitable for quality wine production, port wines, sparkling wines and rose wines. May present aromas of red and ripped fruits, with some floral tones. The velvet color is prevalence, and the wine has good astringency and ability to evolve in the bottle. (Chaves, 2012).

2.4.2 White Varieties

2.4.2.1 Alvarinho

A very Old Portuguese white variety with low production, mainly planted in the areas of Monção and Melgaço (Vinho Verde regions). It is possible to find two distinct forms of this variety; small bunches, compact with small and golden berries, or medium/larger berries that remains greenish when they are ripped. The wines produced from this grape variety are very aromatic, with instrumental analysis showing that chemical compounds contributing the most to the related sensorial perception are fruity (ethyl esters and acetates) and floral aromas (monoterpenes). (Vilanova *et al.*, 2010, Chaves, 2012).

2.4.2.2 Arinto

Cultivated in almost all wine regions in Portugal, being very versatile. In the region of Vinho Verde is known as Pedernã. However, in Bucelas region this variety became well known, being considered the "queen" variety of the region. The variety is with big and compact bunches, composed of small berries. Often used in the production of blended wines and sparkling wines. In Bucelas region, wines produced only from this grape present high acidity, with citrus, floral and fruity aromas when the wine is young (Chaves, 2012)**.** According to Ferreira *et al.,* (2011), the grape variety Arinto received higher score than

Sauvignon Blanc for grape fruit notes by a panel of 10 trained tasters, with the detection of the presence of 4MMP and 3MH in two subsequent years (2009 and 2010) and the presence of 3MHA one of the years (2010)

2.4.2.3 Encruzado

Almost exclusive to the region of Dão, and probably the most used variety there, for white wines; as one variety wine or as a blend. Characterized with good production scale and well balanced in terms of sugar and acidity. On the other hand, it is very sensitive to rot and to harsh weather (rain and wind). The wines produced from it are very aromatic and sharp. Can be saved in a bottle for long periods of time (Chaves, 2012)**.**

2.4.2.4 Viosinho

Only grown in the Douro and Tras-os-Montes, where it has been used since the nineteenth century. Exhibit good quality wines and suitable for the production of still wine and port, but has a low income and so it is little cultivated. The Viosinho has small bunches and early maturing berries but it is very sensitive to rot. Well adopted and develops best in dry soils. The variety produces well-structured wines with fresh and complex floral aromas. Usually they are also alcoholic and able to age in the bottle for a few years (Chaves, 2012). Accoring to Ferreira *et al.,* (2011), the presence of 4MMP and 3MH was detected by analytical methods, though in a panel of 10 tasters, non-of the attributes for this wine were higher than that of Sauvignon Blanc wine.

The Objective of this study is to evaluate a new enological strategy to release volatile thiols from their precursors using a β-lyase enzyme in white and red wines. This approach may permits a wine making style which is independent of the use of selected yeast for the release of thiols compounds.

This is a second study done with this approach, following a study that was done by Chaves, (2012) as a thesis dissertation in ISA, with the main difference been the skin contact time to fclitiatae the extraction of precursors, as described in section 3.

3. MATERIALS AND METHODS

3.1 Vinification process

Two red varieties (Syrah and Touriga Nacional) and four white varieties (Alvarinho, Arinto, Encruzado and Viosinho) were used for this study. All of the varieties are from Instituto Superior de Agronomia's (ISA) vineyards, located in Lisbon, Portugal. Both red and white grapes were monitored for their maturation process close to harvest time (table 5 and 6 respectively). The parameters that were checked for red grapes are; berries weight (g), volume of the must (mL), Brix (%), expected alcohol (% volume), pH, total acids (g/L of tartaric acid), total anthocyanins (mg/L) and total phenols (absorbance units. i.e. a.u). For the white grapes; Brix (%), expected alcohol (% volume), pH, total acids (g/L of tartaric acid). Protocols were adjusted to both white and red wines (see sections 3.1.1 and 3.1.2 respectively), with the use of β-lyase enzyme (Endozym Thiol® for the white wines and Endozyme Thiol Rouge® for the reds), manufactured by AEB group as well as other enological additives, with the aim to promote the completion of the fermentation with an increase in precursors and their free thiols. The β-lyase enzyme is a liquid pectolytic preparation produced with specific secondary activities, promoting the hydrolysis of the thiol's precursors such as 3MH, 3MH, 4MMP and 4MMPOH, from cysteine and glutathione derivatives.

3.1.1 White wines vinification

Viosinho, Alvarinho, Encruzado and Arinto grapes were harvested by hand on the 20/08/2014, 27/08/2014, 05/09/2014 and 23/09/2014 respectively. All the grapes started the processing at the same day of harvesting; crushing of the grapes with the application of potassium metabisulfite, 100 mg/kg of grapes been equivalent to 50 mg Sulphur dioxide, i.e. SO_2 (see section 3.2.1 for further explanation), and an enological product containing SO_2 and ascorbic acid (Aromax B4®) - 50 g/100kg of grapes, with the aim of protecting the must from oxidation. The grape's must was checked for the initial density (g/L at 20 °C), temperature (°C), pH, total acidity (g/L tartaric acid), expected alcohol (% vol.) and Brix (%) (Table 7) and was treated with a pectolytic enzyme (Endozyme ICS 10 Arôme®) 0.6 ml/100kg to induce the partial lysis of the cellular walls and to facilitate the extraction of varietal aromas from the skin to the must, as well as decreasing the viscosity of the must. The must was then macerated for 4 hours at room temperature (skin contact) to further promote extraction of thiols precursors. That was followed by the pressing of the grapes (manual pressing) and the separation of juice and solids. A second pectolytical enzyme was added to the separated juice (Endozyme Muscat®) - 2 g/hl to improve the clarification and extraction. The must was then put for 24 h in room temperature and was racked. After the racking, the must was inoculated with active dry yeast (Zymasil ® Bayanus) - 20 g/hl, obtained from a pure culture of *Saccharomyces bayanus*. This neutral yeast allows a better

evaluation of the β-lyase enzyme additive rather than of the yeast's. The reactivation medium of the yeasts was added with amino acids and vitamins (Fermoplus Energy Glu® - 25% of the amount of the yeast) and was put into the must. 30 g/hl of Nitrogen, vitamins and micro-elements (Enovit®), with 10 g/hl being equivalent to 20 mg/l of assimilated nitrogen was added to the must after inoculation. At the second day of fermentation 5 ml/hl of β-lyase enzyme (Endozyme Thiol®), suitable for white wines was added to the tested wines, leaving the control wines without this last addition. The temperature was kept not less than 18 and no more than 26 °C throughout the fermentation (room temperature), and the densities were measured to control the process of fermentation (table 8). At the last third of fermentation (with density being 1020 to 1040) 30-40 g/hl of nutrients were added to promote a complete fermentation (Fermoplus Integrateur®). Once the fermentation ended, the wine was racked and sampled to check and control its chemical state (section 3.2.1). On the 23/02/2015 the wines were bottled using a manual bottling equipment. Figure 5 illustrates the vinification process of the white wines). Table 25 in the annex summarize the different additives used in the vinification process.

3.1.2 Red wines vinification

Syrah and Touriga Nacional were harvested on the 17/09/2014 and 18/09/2014 respectively. All the grapes started the processing at the same day of harvesting; destemming together with crushing of the grapes and the application of 100 mg/kg potassium metabisulfite, been equivalent to 50 mg of SO_2. The must was then inoculated with active dry yeast (Zymasil® Bayanus) 20 g/hl with a reactivation medium (Fermoplus Energy Glu®) - 25% of the amount of the yeast. The inoculated must was added with Nitrogen, vitamins and micro-elements (Enovit®), and was let fermenting with its solids parts. At the second day of fermentation 5 ml/hl of β-lyase enzyme that is specific for red wines (Endozyme Thiol Rouge®) was added to the tested wines, leaving the control wines without this last addition. The temperature was kept not less than 21 and no more than 26 °C throughout the fermentation (room temperature), and the densities with temperatures were measured to control the process of fermentation (table 8).

At the last third of fermentation (with density being 1020 to 1040) 30-40 g/hl of nutrients were added to promote a complete fermentation (Fermoplus Integrateur®).

Once the fermentation ended, the wine was macerated still to complete a period of about 15 days of maceration, the wine was pressed and separated from its solids. All of the red wines went through Malo-Lactic fermentation (MLF) and afterwards were checked for their basic control parameters (see section 3.2.1) and corrected if needed. The red wines went through the same chemical control as the white wines from the start of vinification (together with MLF control), and were bottled at the same date, 23/02/2015. Figure 6 illustrates the vinification process of the red wines.

Table 25 in the annex summarize the different additives used in the vinification process.

Figure 5: General Flowchart of the main steps for white wines vinification.

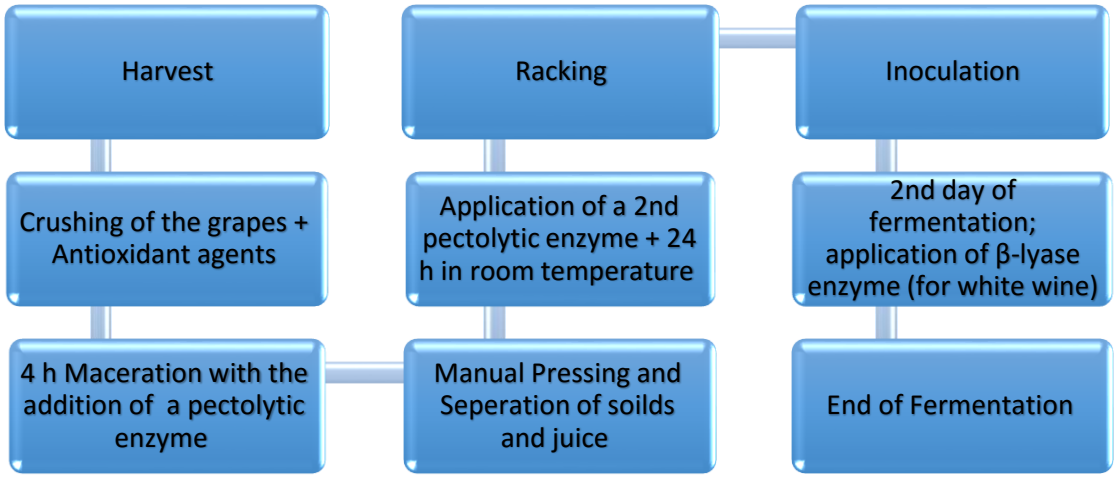
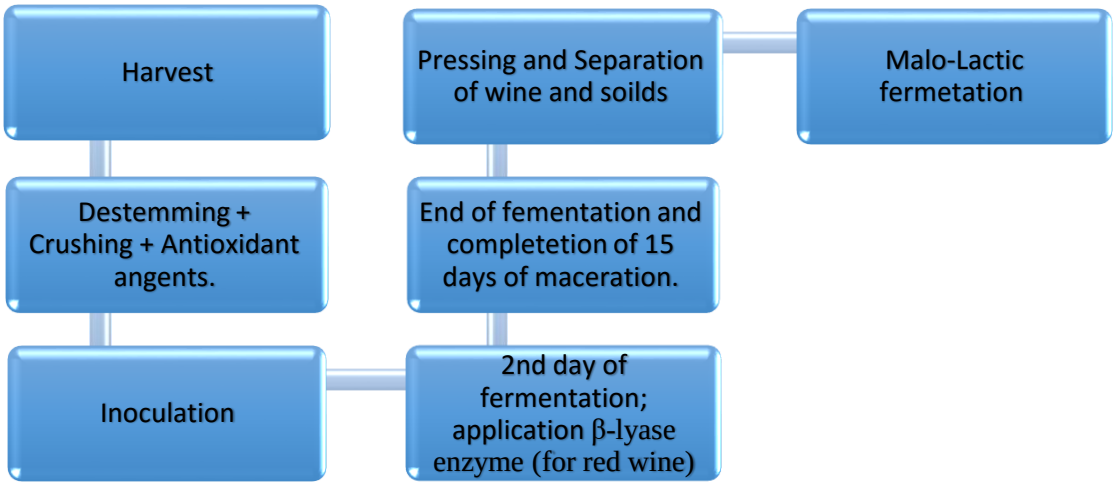


Figure 6: General Flowchart of the main steps for red wines vinification.



3.2 Chemical analysis

3.2.1 Wine's basic parameters

Both red and white wines were analyzed using chemical analysis as mentioned in section 3.1.1 at the receiving of the grapes for the assessment of the must. At the end of fermentation (table 9 for the red wines and table 10 for the whites). The wines were checked for the pH, total acidity, volatile acidity, residual sugar, total Sulfites levels as well as the free levels, which according to it sulfites additions and corrections were done (units are written in the tables) . The wine's sulfur dioxide levels were corrected to achieve around 30 mg/L free SO_2 for the red wines, and about 35 mg/L for the white wines. The form of sulfur dioxide used was potassium metabisulfite ($K_2S_2O_5$) powder diluted with water before use. 100 mg of $K_2S_2O_5$ originates (by hydrolysis) 50 mg of SO2; about 1/3 stays free while the other 2/3 combine. The wines were checked again, about 2 months after fermentation ended for pH, volatile acidity, total Sulfites levels as well as the free levels, which according to it sulfites additions and corrections were done. After bottling the wines were checked again for the same parameters. All of the analysis, during and after fermentation, for the basic control parameters were performed at the laboratories of ISA, according to OIV recommended protocols. The tested basic parameters and their corresponding OIV reference number (or method) are presented in table 2. None of the wines showed any signs of sluggish fermentation. In addition to that, the general chromatic characteristics of the wine were obtained to paint a more comprehensive picture of the wine, together with the phenolic profile and the tannin power.

Parameter	OIV reference	Comments
pH	MA-E-AS313-15	
Sulfur dioxide	OIV-MA-AS323-04B	Free and Total
Total acidity	OIV-MA-AS313-01	
Volatile acidity	OIV-MA-AS313-02	
Alcohol level	OIV-MA-AS312-01B	
Sugar	OIV-MA-AS31101A	
MLF*	Verification of malolactic fermentation by paper chromatography	

Table 2: Control Parameters and their OIV references or method.

*MLF – Malo-Lactic Fermentation.

Analysis of chromatic characteristics:

The chromatic analysis of the wine was done in order to achieve a better and more detailed picture of the wines, both treated and untreated. All the analysis were performed at the laboratories of ISA using

spectrophotometer method proposed by Somers and Evans (1977). The red wines were checked and compared for color intensity (a.u), color shade (a.u), total pigments (a.u), polymerized pigments (a.u), total anthocyanins (mg/l), ionized (colored) anthocyanins (mg/l), degree of ionization of anthocyanin (%), polymerization index (%) as well as total phenols index, total flavonoids and total non-flavonoids. The white wines were checked for color intensity (a.u), total phenols index, total flavonoids and total non-flavonoids. The last three were analyzed using the method suggested by Kramling and Singleton (1969) and the results were expressed as Gallic acid (mg/L) using the curve X= (0.0169 + y)/0.0309, with y being the absorbance value and X the expressed result for total phenols and total non-flavonoids while total flavonoids was expressed in mg/L Catechin using the curve X=y/0.0125. All the trials were done in a triplicate way in 1 mm cell (in that case the dilution factor is X10) or 10 mm cell (which was multiplied by 101) and the formula of each one is presented in table 3, without the dilution factor.

Characteristics	**Formula**
Color intensity*	$A_{420} + A_{520} + A_{620}$
Color Shade	A_{420} / A_{520}
Total Pigments	$A_{520(HCl)}$
Polymerized Pigments	$A_{520(SO2)}$
Total Anthocyanin (mg/L)	$20 \times (A_{520(HCl)} - 5/3A_{520(SO2)})$
Colored Anthocyanin (mg/L)	$20 \times (A_{520} - A_{520(SO2)})$
Ionization Index %	$\frac{100 \times (A_{520} - A5_{20(SO2)})}{(A520_{HCl} - 5/3A_{520(SO2)})}$
Polymerization Index %	$\frac{100 \times (A_{520(SO2)})}{(A_{520(HCl)})}$
Total Phenols index (TPI)	A_{280}
Total Not Flavonoids	$A_{280(\text{with HCl})}$
Total Flavonoids	(TPI – Total Not Flavonoids)

Table 3: Analytical data for the calculation of chromatic characteristics.
* For white wines the intensity is only the measurement of $A_{420.}$

Tannin Power

The Tannin power was evaluated using the method of Freitas and Mateus, (2001). Model solution for dilution of wine sample was prepared by hydro-alcoholic solution of 12% (v/v); pH 3.2, pH was reduced by using tartaric acid solution of 5 g/L. Solution was filtered (0.45 µm) prior to use. Wine sample after centrifugation was diluted by using model solution to 1:50 for red wines and 5:50 for whites. The turbidity of this sample was measured by using the turbidimeter (Hach 2100N). The

Instrument was calibrated by using formazin standards. Later in other test tube diluted wine sample (8 mL) was introduced by 300 µL of BSA (Bovine Serum Albumin 0.8 g/L). It was mixed by using vortex and stored in dark place at ambient temperature for 45 minutes. After that turbidity of this wine sample along with BSA was also measured by using Turbidimeter. Tannin power of wine sample was obtained by using following formula:

$$\text{Tannin Power (NTU/mL)} = (d - d0) / 0.08$$

Where, d - turbidity of sample along with BSA

d0 - turbidity of sample without BSA

3.3 Sensorial Analysis

The sensorial tasting of the wines were held in ISA's tasting room, and was concluded from a panel of 10 trained and experience tasters. Each wine received a random identification code (table 4) and all wines were served randomly. Each taster received a tasting sheet; one for the white wines (Figure 6) and another one for the red wines (Figure 7). The tasters were asked to evaluate the wines in terms of three parameters; color, aroma and taste. Each parameter was further divided into sub-parameters as can be seen in Figures 6 and 7. The sub-parameter "Tropical Notes" was emphasized in this study, and was grouped as passion fruit, grapefruit or cat urine. The evaluation was done on a scale of one to five; one been nonexistent; 2, slightly intense; 3, moderately intense; 4, intense and 5, very intense. For the parameters, equilibrium (aroma and taste) and Overall Assessment; 1, Mediocre; 2, Satisfactory; 3, Good; 4; Very Good; 5, Excellent.

	Code	Variety	Sample
Red Wines	273	Touriga Nacional	Control
	220	Syrah	Test
	171	Syrah	Control
	589	Touriga Nacional	Test
White Wines	121	Alvarinho	Control
	360	Encruzado	Test
	145	Viosinho	Test
	476	Encruzado	Control
	810	Arinto	Test
	160	Viosinho	Control
	190	Alvarinho	Test
	730	Arinto	Control

Table 4: The randomized codes that were given to the wines for the sensorial analysis.

Figure 7: Wine tasting sheet used for the white wines, English and Portuguese versions.

Instituto Superior de Agronomia
White Wine's Tasting Sheet

Name:

Taste the wines in random order and classify the different attributes using the following scales:

For color, aroma and taste: 1. None 2. Slightly Intense (a)
3. Moderately Intense (a) 4. Intense (a) 5. Very Intense (a)

For equilibrium (aroma and taste) and Overall Assessment: 1. Mediocre
2. Satisfactory 3. Good 4. Very Good 5. Excellent

		Wines / Codes							
		121	360	145	476	810	160	190	730
Color	Limpidity								
	Yellow								
	Green								
Aroma	Fruity								
	Floral								
	Tropical Notes*								
	Vegetable								
	Equilibrium								
Taste	Body								
	bitterness								
	Acid								
	Persistence								
	Equilibrium								
Overall Assessment									

***Tropical Notes: Passion Fruit, Grapefruit, Cat Urine.**

Comments:

Instituto Superior de Agronomia
Ficha de Prova de Vinhos Brancos

Nome:

Prove os vinhosem ordem aleatória e classifique os diferentes atributos utilizando as seguintes escalas:

Para Cor, Aroma e Gosto: 1. Inexistente **2.** Pouco Intenso(a)
3. Medianamente Intenso(a) **4.** Intenso(a) **5.** Muito Intenso(a)

Para Equilíbrio (Aroma e Gosto) e Apreciação Global: 1. Medíocre
2. Satisfatório **3.** Bom **4.** Muito Bom **5.** Excelente

		Vinhos / Códigos							
		121	360	145	476	810	160	190	730
Côr	Limpidez								
	Amarelo								
	Verde								
Aroma	Frutado								
	Floral								
	Notas Tropicais*								
	Vegetal								
	Equilíbrio								
Gosto	Corpo								
	Amargo								
	Acidez								
	Persistência								
	Equilíbrio								
Apreciação Global									

***Notas Tropicais: Leia-se, Maracujá, Toranja, Xixi de Gato.**

Observações:

Figure 8: Wine tasting sheet used for the red wines, English and Portuguese versions.

Instituto Superior de Agronomia
Red Wine's Tasting Sheet

Name:

Taste the wines in random order and classify the different attributes using the following scales:

For color, aroma and taste: 1. None 2. Slightly Intense (a)
3. Moderately Intense (a) 4. Intense (a) 5. Very Intense (a)

For equilibrium (aroma and taste) and Overall Assessment: 1. Mediocre
2. Satisfactory 3. Good 4. Very Good 5. Excellent

		Wines / Codes			
		273	220	171	589
Color	Limpidity				
	Red				
	Violet				
Aroma	Fruity				
	Floral				
	Tropical Notes*				
	Vegetable				
	Equilibrium				
Taste	Body				
	bitterness				
	Acid				
	Persistence				
	Equilibrium				
Overall Assessment					

***Tropical Notes: Passion Fruit, Grapefruit, Cat Urine.**

Comments:

Instituto Superior de Agronomia
Ficha de Prova de Vinhos Tintos

Nome:

Prove os vinhosem ordem aleatória e classifique os diferentes atributos utilizando as seguintes escalas:

Para Cor, Aroma e Gosto: 1. Inexistente **2.** Pouco Intenso(a)
3. Medianamente Intenso(a) **4.** Intenso(a) **5.** Muito Intenso(a)

Para Equilíbrio (Aroma e Gosto) e Apreciação Global: 1. Medíocre
2. Satisfatório **3.** Bom **4.** Muito Bom **5.** Excelente

		Vinhos / Códigos			
		273	220	171	589
Côr	Limpidez				
	Vermelho				
	Violeta				
Aroma	Frutado				
	Floral				
	Notas Tropicais*				
	Vegetal				
	Equilíbrio				
Gosto	Corpo				
	Amargo				
	Acidez				
	Persistência				
	Equilíbrio				
Apreciação Global					

***Notas Tropicais: Leia-se, Maracujá, Toranja, Xixi de Gato.**

Observações:

3.4 Statistical Analysis

Each Varietal wine was compared between the control and the tested wine. For the analysis of the chromatic parameters a *t-Test: Paired Two Samples for Means* was performed using Excel. The t-Test allows the comparison of groups with 3 means, as in that case and provides a statistical tool to compare these groups.

A one-way ANOVA (Analysis of Variance) was conducted using RStudio to evaluate any significant differences between the parameters of the wine tasting. All the parameters were evaluated using α=0.01, 0.05 and 0.1 which are automatically generated by the program.

And finally, a Principal Component Analysis (PCA) was utilized on the wine tasting data using Statistica Program, together with Cluster Analysis, to evaluate correlations and possible trends or grouping of the wines.

4. Results and Discussion

4.1 Maturation Control and Basic Parameters

Table 5 (red grapes) and table 6 (white grapes) present the different factors checked to evaluate the maturation of the grapes. Each of the last values for each variety corresponds with the day of harvest for that variety, i.e. Syrah (table 5) was last checked for maturation control on the 17/09/14, which is also its harvest day. The last result for Touriga Nacional for total anthocyanins (716.92 mg/L) is likely represent a problem with the sample of grapes that was collected (not homogeneous sample) since the results are too low.

Variety	Date	Weight of the berries (g)	Vol. of must (mL)	Brix (%)	Expected Alcohol Strength (% Vol.)	pH	Total Acidity (g/L tartaric acid)	Total Anthocyanins (mg/L)	Total Phenols (a.u)
Touriga Nacional	04/08/14	206.5	125.0	16.2	9.5	3.11	12.5	741.54	41.4
	12/08/14	200.3	132.0	17.2	10.1	3.11	9.9	1207.69	55.8
	20/08/14	200.9	118.0	19.6	11.5	3.14	6.9	1709.23	76.8
	27/08/14	200.5	110.0	21.9	12.9	3.35	6.0	2504.62	106.7
	01/09/14	200.5	119.0	22.4	13.2	3.43	5.3	2095.38	81.6
	09/09/14	200.4	120.0	22.8	13.4	3.57	5.1	2270.77	96.7
	18/09/14	187.8	124.0	23.8	14.0	3.58	5.0	716.92	36.1
Syrah	04/08/14	201.1	125.0	15.6	9.2	3.16	13.5	793.08	45.1
	12/80/14	200.2	112.0	17.7	10.4	3.24	10.8	1250.77	48.2
	20/80/14	200.8	122.0	22.1	13.0	3.24	7.4	1549.23	59.5
	27/08/14	200.0	125.0	21.5	12.6	3.21	7.5	1409.23	51.0
	01/09/14	200.2	112.0	22.6	13.3	3.32	6.6	1643.08	60.9
	09/09/14	200.1	126.0	23.5	13.8	3.4	6.5	1643.08	58.9
	17/09/14	200.5	130.0	24.5	14.4	3.57	4.8	1540.00	55.9

Table 5: Maturity control data for the red grapes varieties.
*absorbance units: a.u.

This study does not focus on the maturation parameters that may impact the precursor's levels up to the harvest day, though when looking at the grape berry growth and development, it is possible to notice two sigmoidal growth periods separated by a lag phase; berry formation and berry ripening, respectively (Coombe & McCarthy*.,* 2000). At the second stage of the development we may see the formation and increase in second metabolites, such as volatile flavor compounds. The aroma compounds, or their precursors, are found in the flash, and more considerably in the skin of the berry (González-Barreiro *et al.,* 2015). As mentioned, research done on cysteine precursors, in Sauvignons Blanc grapes from Bordeaux for two years where the level of Cys-4MMP, Cys-4MMPOH and Cys-3MH were checked one month before harvest had shown vast differences in concentration; ripening had a direct effect on processors level by increasing it. (Roland *et al.,* 2011b). Also viticulture practices may change the concentration of the precursors as described in section 2.2.3. The grapes in this research were harvested from the same vineyard where they were subjected to the same management.

Table 7 present the initial must parameters of each of the white and red varieties. All the white and red wines were fermented as described at sections 3.1.1 and 3.1.2 respectively, without problems; sluggish or stopped fermentation. At that stage the wines are still not treated, as they were all added with the β-lyase enzyme on the second day of fermentation.

Variety	Date	Brix (%)	Expected Alcohol Strength (%vol.)	pH	Total Acidity (g/L tartaric acid)
Viosinho	30/07/14	18.7	11.0	3.22	11.0
	07/08/14	21.1	12.4	3.24	7.2
	13/08/14	22.5	13.2	3.38	6.0
	20/08/14	23.0	13.5	3.28	5.1
Arinto	30/07/14	9.1	5.4	3.02	31.2
	13/08/14	13.7	8.1	3.04	20.6
	20/08/14	17.3	10.2	2.95	14.4
	27/08/14	18.6	10.9	3.15	13.1
	03/09/14	20.7	12.2	3.21	10.1
	09/09/14	20.3	11.9	3.37	8.7
	19/09/14	20.9	12.3	3.18	7.8
	23/09/14	21.5	12.6	3.23	7.8
Encruzado	30/07/14	15.4	9.1	3.13	14.9
	13/08/14	18.3	10.8	3.17	9.3
	20/08/14	19	11.2	3.23	6.8
	27/08/14	20.6	12.1	3.27	6.3
	05/09/14	20.5	12.1	3.27	6.0
Alvarinho	30/07/14	15.8	9.3	3.07	21.5
	13/08/14	20.2	11.9	3.14	12.2
	20/08/14	21.8	12.8	3.06	9.8
	27/08/14	22.1	13.0	3.17	8.6

Table 6: Maturity control for white grapes varieties.

Variety	Date	Brix (%)	Excepted Alcohol (% vol.)	pH	Total Acidity (g/L tartaric acid)	Density (g/L at 20 °C)	Temp. (°C)
Viosinho	20/08/14	22.8	13.4	3.38	5.4	1102	25
Alvarinho	27/08/14	22.6	13.3	3.35	8.1	1097	24
Encruzado	05/09/14	20.3	11.9	3.39	5.9	1088	22
Arinto	23/09/14	19.6	11.5	3.36	5.9	1085	22
Syrah	17/09/14	22.3	13.1	3.45	5.0	1101	25
Touriga Nacional	18/09/14	26.0	15.3	3.63	5.6	1114	23

Table 7: Initial must parameters of each of the white and red varieties.

Variety	Sample	Density (g/L at 20 °C)		Temperature (°C)	
		Initial	Final	Initial	Final
Syrah	Control	1101	994	25	22
	Endozym Thiol Rouge	1101	994	25	22
Touriga Nacional	Control	1114	995	23	22
	Endozym Thiol Rouge	1114	996	23	22
Arinto	Control	1085	995	22	22
	Endozym Thiol	1085	995	22	22
Viosinho	Control	1102	992	25	24
	Endozym Thiol	1102	992	25	24
Alvarinho	Control	1097	994	24	24
	Endozym Thiol	1097	994	24	24
Encruzado	Control	1088	992	22	23
	Endozym Thiol	1088	993	22	23

Table 8: Temperatures and Densities in the beginning and the ending of fermentation.

As described in section 2.2.4, in trials done by Peyrot des Gachons *et al.,* (2002) with skin contact of Sauvignon Blanc grapes, an increase in thiols precursors in the juice was observed, showing a favorable results for Cys3-MH, with little effect on Cys-4MMPOH and Cys-4MMP. With that, enological additives in the form of pectolytic enzymes were added to the must to facilitate the extraction of the precursors as described in vinification process of section 3.1. Additionally, an increase in the temperature during the skin contact had resulted in a considerable increase in Cys-3MH in the must, but allowed only a slight increase in 4MMPOH and 4MMP precursors (Peyrot des Gachons *et al.,* 2002). All of the wine's protocols were conducted to felicitate an extraction of precursors by permitting more skin contact time (i.e. maceration) and appropriate temperature. The protocol for the white wines provided 4 hours of maceration time, while the red grapes were fermented with their solids (berry's skin) and then were left for about 15 days after fermentation. The initial and final temperatures are presented in table 8. The range of temperatures for the red wines was 21 to 26 °C and for the white wines 18 to 26 °C. Studies done on Sauvignon Blanc wines had shown that the final concentrations of the three main volatile thiols (4MMP, 3MH and 3MHA) were higher when the temperature during fermentation was higher (20 °C compare to 13 °C). (Masneuf-Pomarède *et al.,* 2006). The basic control parameters to evaluate the state of the wines at the end of fermentation and during the ageing process, for both red and white wines (tables 9 and 10 respectively), suggest a good fermentation process, the sugar levels agree with the expected values for dry wines. Looking at both red and white wines we can see three different dates in which the wines were monitored and tested; the first time is the date in which fermentation ended, second time was at the ageing process of the wine, i.e. after fermentation and prior to bottling and last time was after the bottling of the wine. OIV standards state that red wines

pH values range from 2.9 to 4.2. All of The pH values showed an increase from the end of fermentation to after the bottling of the wines, as expected, especially with all of the red wines completing malo-lactic fermentation (MLF). The highest pH value was 4.00 for Touriga Nacional and 3.80 for Syrah (table 9). Both in agreement with the expected range, though can be considered a bit high. Wine's chemical and biological stability are very dependent on pH value; the wines were corrected for the SO_2 levels to maintain biological stability and to try and prevent any oxidation. The correction was done with potassium metabisulfite ($K_2S_2O_5$) powder diluted with water before use. 100 mg of $K_2S_2O_5$ originates (by hydrolysis) 50 mg of SO2; about 1/3 stays free while the other 2/3 combine. For the red wines the objective was to achieve about 30 mg/L Free SO_2. The volatile acidity levels are an important indicator for microbiological contamination in the wine and high levels of it may damage the organoleptic sensation of it. Looking at the results in table 9 (for the red wines) we can see that all of the wines present acceptable levels, i.e. below 1.1 g/L of acetic acid (Ribéreau-Gayon *et al.*, 2006), suggesting a well protection process during the vinification and ageing. From a higher point of view, we can not only observe the stability of the wines throughout the process of vinification, but also notice that all parameters are in the expected range; both for treated and untreated wines, concluding that up to this stage, the treatment been tested i.e. the addition of β-lyase enzyme, had no effect (that is above the legal regulations) on these parameters for the red wines.

Variety	Date	Sample	Volatile Acidity (g/L acetic acid)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	pH	Alcohol content at 20ºC (%Vol.)	Residual Sugar (g/L)	Total Acidity (g/L tartaric acid)	MLF*
Touriga Nacional	03/10/14	Control	0.58	22	67	3.77	16.2	1.5	5.9	02/12/14
	03/10/14	Endozyme Thiol Rouge	0.59	16	64	3.76	16.2	1.8	6.2	09/12/14
	20/11/14	Control	0.66	6	20	4.03	-----	-----	-----	-----
	20/11/14	Endozyme Thiol Rouge	0.66	6	15	4.00	-----	-----	-----	-----
	20/02/15	Control	0.60	40	85	3.99	-----	-----	-----	-----
	20/02/15	Endozyme Thiol Rouge	0.58	46	82	3.96	-----	-----	-----	-----
Syrah	03/10/14	Control	0.25	16	51	3.62	14.3	1.1	5.7	18/11/14
	03/10/14	Endozyme Thiol Rouge	0.42	19	61	3.63	14.2	1.1	5.7	18/11/14
	20/11/14	Control	0.51	10	10	3.80	-----	-----	-----	-----
	20/11/14	Endozyme Thiol Rouge	0.57	4	10	3.77	-----	-----	-----	-----
	20/02/15	Control	0.48	44	97	3.74	-----	-----	-----	-----
	20/02/15	Endozyme Thiol Rouge	0.48	44	72	3.74	-----	-----	-----	-----

Table 9: Basic control Parameters for red wines at the end of fermentation and during the ageing process. (*The MLF; Malo-Lactic fermentation, refers to the date in which the verification of the termination of malolactic fermentation occurred using paper chromatography)

Table 10 presents the same parameters for the white wines (except MLF, which the white wines did no go through), suggesting a similar picture. The pH values are lower than that of the red wines, as expected. All of the volatile acidity values are in accepted range, and corrections of SO_2 were done if needed as described already, with the objective of reaching about 35 mg/L free SO_2. All the wines completed fermentation without problems, and all of the results suggests that there are no differences to the wines due to the treatment, up to this point.

Variety	Date	Sample	Volatile Acidity (g/L acetic acid)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	pH	Alcohol content at 20ºC (%Vol.)	Residual Sugar (g/L)	Total Acidity (g/L tartaric acid)
Viosinho	29/08/14	Control	0.42	32	83	3.39	14.3	0.9	6.3
	29/08/14	Endozyme Thiol	0.51	35	102	3.41	14.2	1.1	6.3
	25/11/14	Control	0.38	11	48	3.42	------	------	------
	25/11/14	Endozyme Thiol	0.38	14	50	3.42	------	------	------
	20/02/15	Control	0.39	29	115	3.36	------	------	------
	20/02/15	Endozyme Thiol	0.39	28	117	3.35	------	------	------
Alvarinho	05/09/14	Control	0.43	29	93	3.23	13.8	1.7	8.7
	05/09/14	Endozyme Thiol	0.53	32	96	3.25	13.8	1.7	9.0
	25/11/14	Control	0.50	25	65	3.26	------	------	------
	25/11/14	Endozyme Thiol	0.47	25	55	3.26	------	------	------
	20/02/15	Control	0.53	30	107	3.20	------	------	------
	20/02/15	Endozyme Thiol	0.51	28	102	3.20	------	------	------
Encruzado	17/09/14	Control	0.32	19	70	3.36	12.7	1.0	6.8
	17/09/14	Endozyme Thiol	0.26	22	74	3.38	12.9	1.2	6.8
	25/11/14	Control	0.23	24	90	3.32	------	------	------
	25/11/14	Endozyme Thiol	0.31	23	88	3.32	------	------	------
	20/02/15	Control	0.34	40	142	3.27	------	------	------
	20/02/15	Endozyme Thiol	0.34	37	140	3.27	------	------	------
Arinto	01/10/14	Control	0.39	51	176	3.30	12.1	1.4	7.4
	01/10/14	Endozyme Thiol	0.41	54	182	3.32	11.9	1.4	7.2
	25/11/14	Control	0.35	39	115	3.32	------	------	------
	25/11/14	Endozyme Thiol	0.35	42	103	3.30	------	------	------
	20/02/15	Control	0.47	36	137	3.25	------	------	------
	20/02/15	Endozyme Thiol	0.49	38	140	3.26	------	------	------

Table 10: Basic control Parameters for white wines at the end of fermentation and during the ageing process.

4.2 Chromatic Characteristics

As mentioned in section 3.2.1, a chromatic profile of both red and white wines was established. The white wines were checked for color intensity, total phenols, total flavonoids and total non-flavonoids (units are in the table). Table 11 presents the results for the different parameters for all of the four grape varieties. The results are an average of triplicate trials (the complete data is presented in table 26 of the annex). All of the results represent the expected values for these grape varieties. The triplicated results were tested using *t-Test: Paired Two Sample for Means*, with significance level (α) of 1 % and 5% to review each of the chromatic parameters, comparing the control sample to that of the treatment. The t-Test allows the comparison of groups with less than 3 means, as in that case and provides a statistical tool to compare these groups. Table 12 provides the P values for the white wines. When P value is higher than 0.05 (i.e. α), than the null hypothesis cannot be rejected with 95% confidence. Which means that the means of the chromatic factor, for both control and treated samples are equal, i.e. taken from the same population/sample and any differences are due to chance.

Variety	Sample	Absorbance at 420 m (a.u)	Total Phenols (a.u)	Total Phenols (mg/L Gallic acid)	Total Flavo. (mg/L Catechin)	Total Non Flavo. (mg/L Gallic acid)
Viosinho	Control	0.147	9.96	322.9	500.27	120.5
	Endozym Thiol	0.139	10.36	335.7	501.1	133.0
Alvarinho	Control	0.192	11.12	360.5	558.1	134.7
	Endozym Thiol	0.157	11.43	370.3	580.5	135.5
Encruzado	Control	0.086	9.89	320.5	522.0	127.5
	Endozym Thiol	0.094	10.34	335.1	532.0	121.0
Arinto	Control	0.233	10.71	347.0	546.4	126.0
	Endozym Thiol	0.228	10.90	353.4	566.9	124.0

Table 11: Chromatic analysis of the white wines, average results.

Looking at the results of table 12 we can see that for most of the parameters, no significant differences is observed, except for that of total phenols for Alvarinho and Encruzado, the means of the control is different and lower than that of the treatment, having P values of 0.0044 (Alvarinho) and 0.0233 (Encruzado), suggesting that the means are different with 99% (α=0.01) and 95% (α=0.05) respectively. The cause for these differences might be as a result of inaccuracies while conducting the experiment,

due to fact that only these two results present a significant difference out of all of the other parameters tested. Though, when considering that both of the significant results suggest a higher phenol content in these treated wines, and that the rest of the wines show differences that are not as significant but with P values of 0.1047 for Viosinho and 0.0698 for Arinto for total phenols, we may consider a trend which is the impact on the phenols due the use of the β-lyase, a pectolytic enzyme. Nevertheless, the results as a general suggest no significant differences between control and treatment with the risk level of 0.05; it can be concluded that the treatment does not have a great impact on these parameters, and a well expected wine's profile is observed.

Variety	Absorbance	Total Phenols	Total Flavo.	Total Non Flavo.
Viosinho	0.2664	0.1047	0.9773	0.1581
Alvarinho	0.1628	0.0044*	0.0593	0.6979
Encruzado	0.0572	0.0233*	0.2686	0.3715
Arinto	0.2663	0.0698	0.0164	0.1835

Table 12: P values of *t-Test: Paired Two Sample for Means*, with significance level α of 1% and 5% for chromatic parameters of the white wines. (*α=0.05, **α=0.01).

The red wines were checked and compared for color intensity, color shade, total pigments, polymerized pigments, total anthocyanins, ionized (colored) anthocyanins, degree of ionization of anthocyanin, polymerization index as well as for total phenols, total flavonoids and non-flavonoids and Tannin power (Table 13). The data presented is the average results of triplicated tests (full data is in table 27 of the annex). The same t-test was conducted and the results are presented in table 14.

When considering Syrah wine, only the results for color intensity (0.0287) and Tannin power (0.0374) shows significant difference with a significant level of α=0.05. As for the Touriga Nacional wines, the parameters color shade (0.0256, α=0.05), Poly. Pigments (0.0082, α=0.01) and total phenols (0.0466, α=0.05) are significantly different. As for the white wines, one may conclude that because these very few random parameters (less than in the white wines) present this kind of difference that the reason lies in measurement error, with the possibility of an effect of the treatment, such as for the case of Tannin power for Syrah.

	Red Varieties / Samples			
	Syrah		Touriga Nacional	
	Control	Endozyme Thiol Rouge	Control	Endozyme Thiol Rouge
Color Intensity (a.u)	9.04	8.67	16.50	17.29
Color Shade (a.u)	0.690	0.692	0.765	0.736
Total Pigment (a.u)	25.86	26.39	35.75	34.34
Polymerized Pigment (a.u)	2.66	2.65	4.85	4.95
Total Anthocyanins (mg/L)	428.6	439.4	553.4	521.9
Colored Anthocyanins (mg/L)	38.8	35.5	60.8	68.9
Ionization Index (%)	9.1	8.1	11.0	13.2
Polymerization index (%)	10.3	10.1	13.6	14.4
Total Phenols (a.u)	42.83	43.10	75.17	73.70
Total Phenols (mg/L Gallic acid)	1386.7	1395.4	2433.1	2385.7
Total Flavo. (mg/L Catechin)	2986.9	3023.2	5476.5	5405.9
Total Non Flavo. (mg/L Gallic acid)	178.4	172.4	217.7	198.9
Tannin Power (NTU/ml)	143.54	158.04	287.83	276.04

Table 13: Chromatic analysis of the red wines, average results.

	Red Varieties / Samples	
	Syrah	Touriga Nacional
Color Intensity	0.0287*	0.1495
Color Shade	0.4226	0.0256*
Total Pigment	0.3948	0.1917
Polymerized Pigment	0.9715	0.0082*
Total Anthocyanins	0.3516	0.9334
Colored Anthocyanins	0.1076	0.2230
Ionization Index	0.9302	0.1040
Polymerization index	0.4226	0.43968
Total Phenols	0.6348	*0.0466
Total Flavo.	0.1450	0.1467
Total Non Flavo.	0.3866	0.1433
Tannin Power	0.0374*	0.6575

Table 14: P values of *t-Test: Paired Two Sample for Means*, with significance level (α) of 1% and 5%, for chromatic parameters of the red wines (*α=0.05).

4.3 Sensorial Analysis results

The wine tasting was held with a panel of 10 experience tasters as described in section 3.3. Table 15 presents the average results for the red wines. The results are presented according to the wines and not the identification codes used for the tasting. The letter C beside the grape variety's name stands for control, while the T stands for test (i.e. treated wines).

		Wines			
		Syrah T	Syrah C	Touriga Nacional T	Touriga Nacional C
Color	Limpidity	3.80	4.10	3.80	3.90
	Red	3.30	3.10	3.40	3.40
	Violet	3.10	3.45	3.80	3.70
Aroma	Fruity	2.80	2.90	3.40	3.70
	Floral	2.00	2.10	2.10	2.60
	Tropical Notes	1.45	1.40	1.15	1.70
	Vegetal	2.00	1.80	1.60	1.60
	Equilibrium	2.50	3.00	3.25	3.10
Taste	Body	3.15	2.95	3.60	3.60
	Bitterness	2.30	2.35	2.20	2.50
	Acids	3.10	2.80	3.25	2.65
	Persistence	3.05	3.15	3.30	3.40
	Equilibrium	2.90	3.10	3.30	3.20
Overall Assessment		2.75	2.85	3.20	3.10

Table 15: Average results of the sensorial tasting for red wines, presented in non-randomized order.

When considering the average results, an immediate observations can be seen regarding the possible trends between the treated wines (T) and the control (C) that is without performing a statistical analysis. Table 16 presents the attributes that showed an increase in average ranking for the treated wines compared with the control, as well as the attributes showing a decrease. For example, if considering Limpidity, for Syrah, we can observe an average score that is lower for the treated wine (3.8) compared with the control (4.1), with a difference of (3.8-4.1= **-** 0.3). In order to understand if the changes presented in table 16 represent a trend that is statistically significant a one way-ANOVA was performed. The null hypothesis is that there is no difference between the control and treatment wines; they are all from the same population and any visible differences are random. The alternative hypothesis is that the means of each group (control and treatment) are not equal and this is due to the treatment. When P value is higher than a given α than we can cannot reject the null hypothesis, suggesting that the tested wines were not influenced by the treatment for a given parameter.

		Wines	
		Syrah	Touriga Nacional
Color	Limpidity	(-0.3) ↓	(-0.1) ↓
	Red	↑ (+0.2)	(0)
	Violet	(-0.35) ↓	(+0.1)
Aroma	Fruity	(-0.1) ↓	(-0.3) ↓
	Floral	(-0.1) ↓	(-0.5) ↓
	Tropical Notes	↑ (+0.05)	(-0.55) ↓
	Vegetal	↑ (+0.2)	(0)
	Equilibrium	(-0.5) ↓	↑ (+0.15)
Taste	Body	↑ (+0.2)	(0)
	Bitterness	(-0.05) ↓	(-0.3) ↓
	Acids	↑ (+0.3)	(+0.6)
	Persistence	(-0.1) ↓	(-0.1) ↓
	Equilibrium	(-0.2) ↓	↑ (+0.1)
Overall Assessment		(-0.1) ↓	↑ (+0.1)

Table 16: The difference between each treated wine to its control wine, with no statistical analysis, red wines.

Table 17 shows the results of the one-way ANOVA; the results presented in the table are the P values (Pr>F) of each attribute as a respond to the treatment. The significance codes that were used were α of 10%, 5% and 1% as they are given automatically when using an ANOVA algorithm on R studio program, though these values are not present in the table since non-of the results suggested a significant difference between the treated wines and the control for the red wines.

		Wines	
		Syrah	Touriga Nacional
Color	Limpidity	0.5375	0.8437
	Red	0.6301	1
	Violet	0.3886	0.8332
Aroma	Fruity	0.8364	0.5305
	Floral	0.7541	0.1373
	Tropical Notes	0.8437	0.1012
	Vegetal	0.6367	1
	Equilibrium	0.1605	0.6319
Taste	Body	0.5172	1
	Bitterness	0.9170	0.5963
	Acids	0.3419	0.0688
	Persistence	0.7287	0.7730
	Equilibrium	0.5520	0.6278
Overall Assessment		0.7567	0.7031

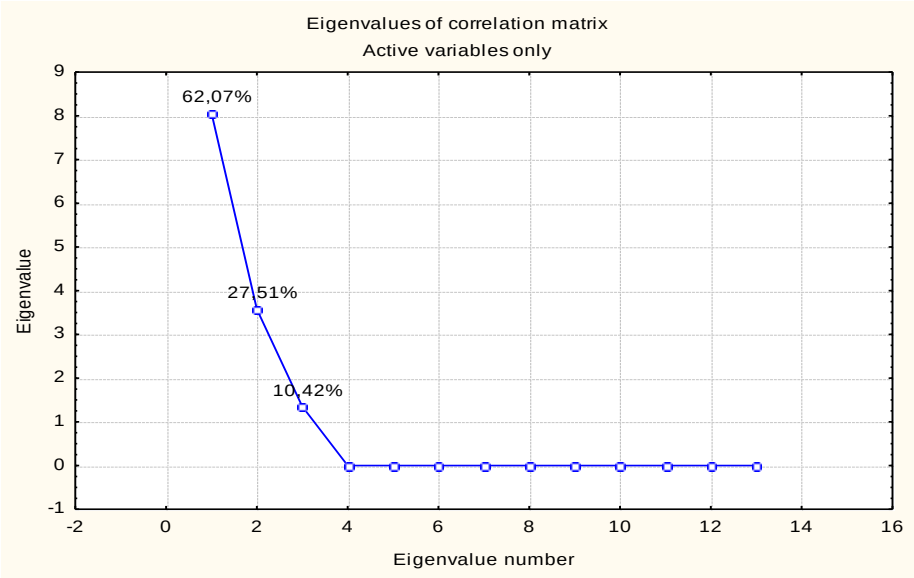
Table 17: Statistical analysis of the red wines tasting panel; one-way ANOVA.

Number of the commentaries that were received in the tasting suggested that the red wines are difficult to differentiate between, which agrees with the statistical results of the ANOVA test. Never the less, it was already shown that blackcurrant aromas may be identified in red wines as a result of high thiols levels (Rigou *et al.*, 2014). In the primary research done by Chaves, (2012) no siginficant differences in the red wine sensorial anaylsis was shown as well, though an analysis of the conentration of the main thiols was done, indicating an incraese in thiols for Touriga Nacional at about double of the treated wines compared with the control. An analysis such as that was not performed yet for the reults presented here, but regardles of it an additional point to consider is that any olfactometry test done with a wine medium does not take into account the synergic or antagonistic effects among the volatile compounds in the wine's matrix that could enhance or depress each other (Roland *et al.*, 2011b). To profile better this kind of relations in the wine medium an additional quantitative approach should be considered, such as the calculation of the concentration to perception threshold ratio (i.e. odor active values, OAV), to try and illustrate the major contributors to the wine's aroma (Roland *et al.*, 2011b, Rigou *et al.*, 2014). This kind of approach will allow to evaluate the concentration of thiols in the wine, the increase due to treatment and the relative contribution of it to the overall aroma. That can be used to determine the potentiality of the variety with regard to its reaction to the treatment.

To better understand the relations of the parameters checked in the sensorial analysis a PCA was performed. The 14 dimensional dataset (14 different attributes) was reduced to 3 dimensional matrix. Looking at the Eigenvalues of the correlation matrix (table 18) we can see that the first value (factor) explains about 62% of the total variance, the second value about 27% and third approximately 10%. When considering the change in slope (graph 1), a distinct change is seen between the second and the third values, suggesting the use of the first two factors for the projection of the variables, which explains about 90% (89.985%) of the total variance.

	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
1	8.069531	62.07332	8.06953	62.0733
2	3.576516	27.51166	11.64605	89.585
3	1.353953	10.41502	13	100

Table 18: Eigenvalues of correlation matrix, and related statistics for red wines, active variables only.

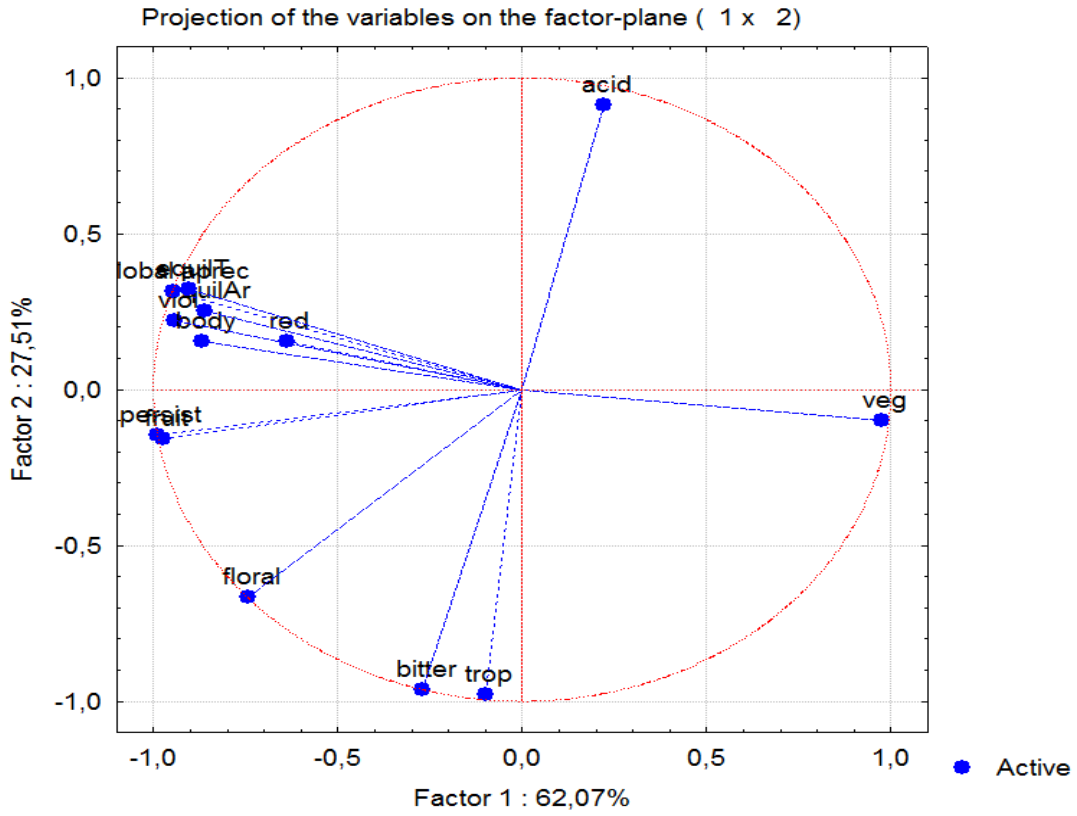


Graph 1: Eigenvalues of correlation matrix as a function of Eigenvalue number for red wines.

Table 19 presents the factor coordinates of the variables, based on correlation with the first and second factors chosen as the principal components for the sensorial analysis of the red wines. The values in each of the boxes represent a correlation coefficient. Those that are indicated by yellow color represent the most negatively correlated parameters with a factor, while the red ones represent the positive correlations. A projection of the variables on the factor plane was conducted, using these first and second factors/dimensions (graph 2).

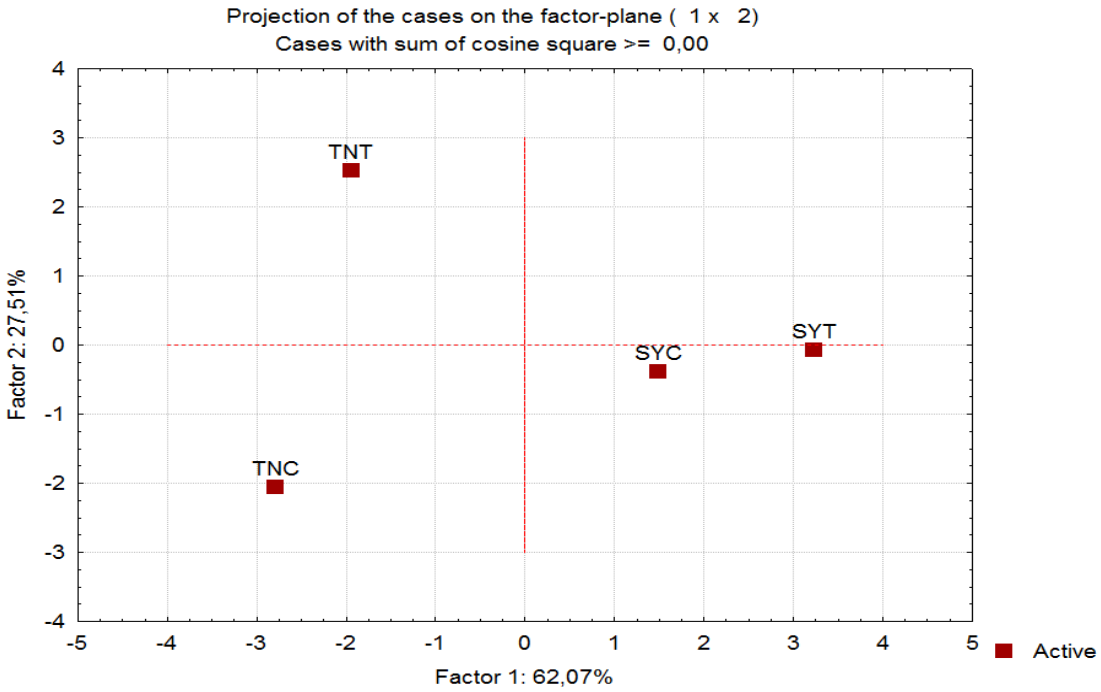
	Parameter	Factor 1	Factor 2
Color	Red	-0.64008	0.154299
	Violet	-0.94338	0.222963
Aroma	Fruity	-0.97404	-0.15675
	Floral	-0.74312	-0.66407
	Tropical notes	-0.09755	-0.97753
	Vegetable	0.975578	-0.09941
	EquilibriumAr	-0.85959	0.250805
Taste	Body	-0.86818	0.15378
	Bitterness	-0.27	-0.96232
	Acid	0.219736	0.913397
	PersistenceT	-0.98933	-0.14559
	Equilibrium	-0.90261	0.323628
	Global ap.	-0.94885	0.314907

Table 19: Factor coordinates of the variables, based on correlations with factor one and two for the red wines.



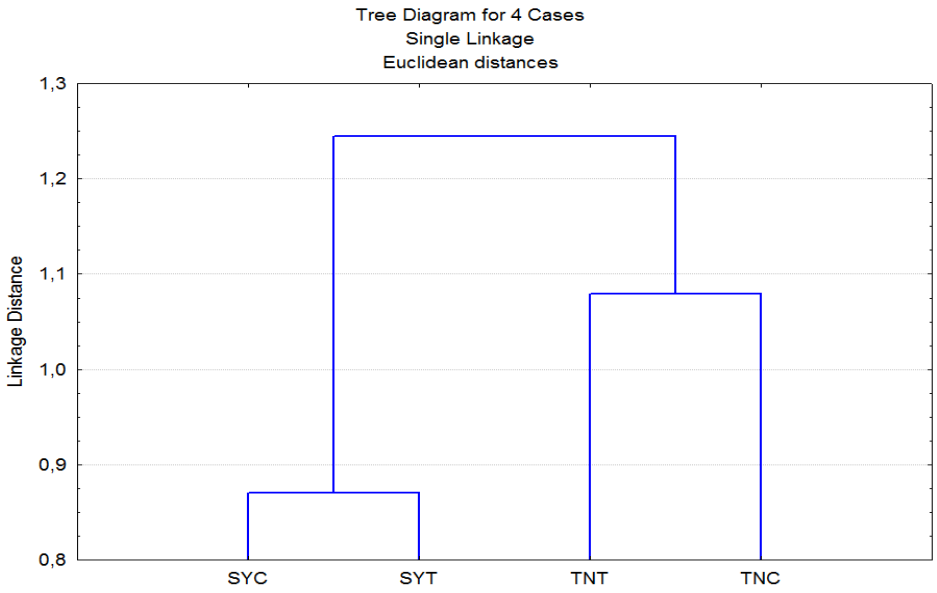
Graph 2: Projection of variables on the factor-plane, using Factor 1 and Factor 2, for the red wines.

The PCA suggests a high positive correlation with factor 1 (axe X) for the parameter vegetable, and a negative correlation with fruity, floral and equilibriumAr (the use of Ar is to differentiate Aroma from the taste's equilibrium written as equilibriumT) in terms of aromas, as well body, persistence and equilibriumT in terms of taste and the color parameters (red and violet). When considering the aroma parameters, as those are of main interest for this research, it seems that wines that are preserved as fruity and floral are differentiated from the vegetable wines, as can be expected. Factor 1, being correlated with this aromatic parameters shows no significant correlation with tropical notes, the major parameter in this research. Nonetheless, tropical notes is highly negatively correlated with factor 2 (together with bitterness), while acidity is positively correlated with factor 2, suggesting that wines that are acid are differentiated form the tropical notes. When projection the cases, i.e. treated and untreated wines (graph 3) we can see the grouping of Syrah wines, both treated (SYT) and control (SYC) on the same axe, that is factor 1, characterized by vegetable aromas, while the Touriga Nacional wines (TNT for treated wine and TNC for control) are characterized more by the floral and fruity notes, with TNC been the more floral one.



Graph 3: Projection of the different red wines (treatment and control), using Factor 1 and Factor 2.

The PCA results supports the ANOVA results of the sensorial analysis, suggesting no noticeable impact of the treatment on the tropical notes of the wines and supports the observation of members of the panel that the red wines are difficult to differentiate between. A further support to that can be seen using a cluster analysis of the different wines (graph 4), which groups the Syrah wines together separately from the Touriga Nacional wines, thus supporting that at this point the enzyme treatment does not result with significant differences and existed differences seems to be due to the cultivator more than treatment. An interesting information that can be seen is that it appear that Touriga Nacional is more influenced by the treatment, observed by a higher linkage distance of the of Syrah (graph 4), which agrees with former results by Chaves, (2012) that showed bigger thiols concentration increase with Touriga Nacional than Syrah.



Graph 4: Cluster analysis for the red wine.

When considering the white wines, the same statistical approach was conducted. A panel of 10 professional tasters evaluated the wines. While the evaluation took place a defect in the Arinto wines was detected by number of tasters, pointing an oxidation defect in both aroma and taste. This defect is most likely a result of air exposure and filtration at or after the bottling of the wine. Nevertheless most of the panel evaluated the wines with all of the parameters, except one member which did not evaluate the aroma and taste of Arinto wines; this was taking into account when performing the statistical analysis of the wines in both ANOVA and PCA.

As for the red wines, table 20 presents the average results of the sensorial tasting for the white wines, while table 21 presents any possible trends, without performing a statistical analysis. A one-way ANOVA was conducted in order to evaluate each of the possible trends and to give it a statistical significances (table 22), using R Studio program.

		Wines							
		Alv. T	Alv. C	Enc. T	Enc. C	Vio. T	Vio. C	Ar. T	Ar. C
Color	Limpidity	4.55	4.2	4.4	4.55	4.4	4.4	4.3	4.45
	Yellow	3.3	3.0	2.5	2.7	2.6	2.8	4.6	4.70
	Green	1.65	2.1	1.95	2.3	2.2	2.2	1.1	1.10
Aroma	Fruity	2.8	3.7	3.0	2.3	3.4	2.9	2.9	2.90
	Floral	2.3	2.4	2.5	2.1	2.1	2.3	2.4	2.10
	Tropical Notes	3.0	3.3	2.1	1.5	2.7	2.4	2.3	2.20
	Vegetable	2.2	2.1	1.8	1.8	1.9	1.5	1.3	1.35
	Equilibrium	3.5	3.7	3.1	2.6	3.3	3.5	2.4	2.10
Taste	Body	3.6	2.95	2.6	2.6	2.8	3.25	3.25	2.95
	Bitterness	2.2	2.25	1.8	1.8	2.0	2.0	2.0	1.80
	Acid	3.75	3.75	2.8	2.7	2.8	3.4	2.9	2.95
	Persistence	3.85	3.4	2.9	2.5	3.0	3.2	2.45	2.75
	Equilibrium	3.7	3.35	2.7	2.5	2.85	3.0	2.6	2.15
Overall Assessment		3.6	3.5	2.85	2.5	2.8	3.15	2.1	2.25

Table 20: Average results of the sensorial tasting for white wines, presented in non-randomized order. (Alvarinho = Alv., Encruzado = Enc., Viosinho = Vio., Arinto = Ar., T=test, C=control).

		Wines			
		Alvarinho	Encruzado	Viosinho	Arinto
Color	Limpidity	(+0.35)	(-0.15)	(0)	(-0.2)
	Yellow	(+0.3)	(-0.2)	(-0.2)	(-0.1)
	Green	(-0.45)	(-0.4)	(0)	(0)
Aroma	Fruity	(-0.9)	(+0.7)	(0.5)	(0)
	Floral	(-0.1)	(+0.4)	(-0.2)	(+0.3)
	Tropical Notes	(-0.3)	(+0.6)	(+0.3)	(+0.1)
	Vegetable	(+0.1)	(0)	(+0.4)	(0)
	Equilibrium	(-0.2)	(+0.5)	(-0.2)	(+0.3)
Taste	Body	(+0.65)	(0)	(-0.45)	(+0.3)
	Bitterness	(-0.1)	(0)	(0)	(+0.2)
	Acid	(0)	(+0.1)	(-0.6)	(-0.1)
	Persistence	(+0.45)	(+0.4)	(-0.2)	(-0.3)
	Equilibrium	(+0.35)	(+0.1)	(-0.15)	(+0.4)
Overall Assessment		(+0.1)	(+0.35)	(-0.35)	(-0.2)

Table 21: The difference between each treated wine to its control wine, with no statistical analysis, white wines.

Looking at the results, most of the parameters do not suggest any significant differences between treated and untreated wines, except the attributes Body for Alvarinho wines with a P value of 0.0181 (p > F at a 5%), indicating a significant difference with 95% confidence (table 22), and Fruity for Encruzado wine, with P value of 0.0041 (p > F at a 1%), indicating a significant difference with 99% confidence (table 22). Both changes in these parameters can be linked to the treatment, thus suggesting that the treated Alvarinho wine has higher results for its Body (table 21) than the control and the Encruzado treated wine is fruiter than the control, having higher results for this parameter (table 21).

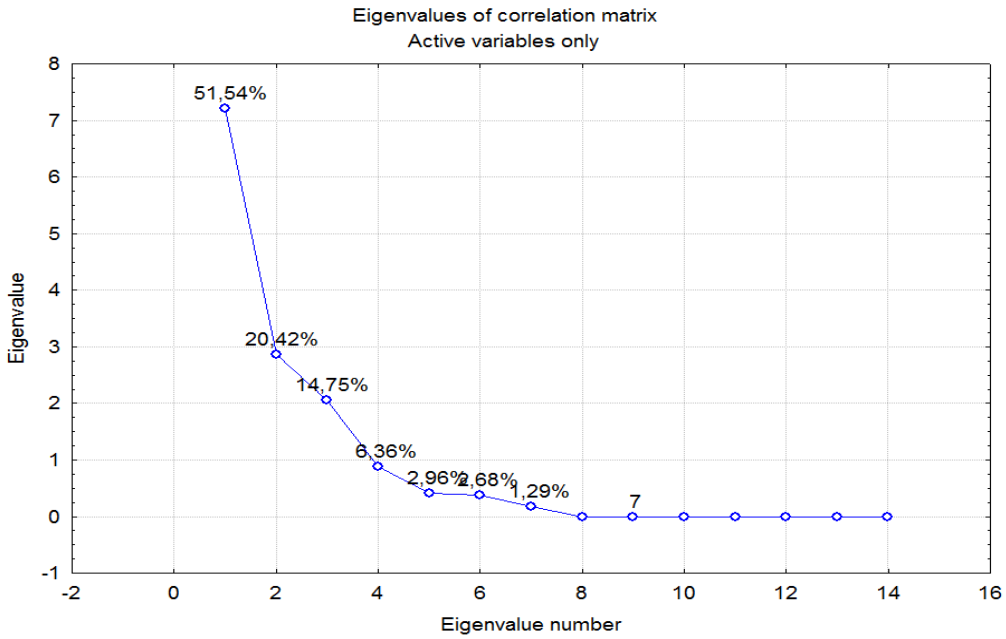
		Wines			
		Alvarinho	Encruzado	Viosinho	Arinto
Color	**Limpidity**	0.3998	0.6813	1	0.7498
	Yellow	0.4872	0.6255	0.6410	0.6944
	Green	0.1992	0.3151	1	0.3322
Aroma	**Fruity**	0.0964	0.0041**	0.2409	1
	Floral	0.8502	0.3750	035743	0.4774
	Tropical Notes	0.4583	0.0798	0.5165	0.8597
	Vegetable	0.8655	1	0.4228	1
	Equilibrium	0.6255	0.1373	0.5673	0.4774
Taste	**Body**	0.0181*	1	0.2188	0.2817
	Bitterness	0.9076	1	1	0.5360
	Acid	1	0.7364	0.1964	1
	Persistence	0.2592	0.1800	0.6132	0.6944
	Equilibrium	0.2593	0.5785	0.6980	0.3791
Overall Assessment		0.7396	0.2263	0.3802	0.5339

Table 22: Statistical analysis of the white wines tasting panel; one-way ANOVA. (*α=0.05, ** α=0.01).

A PCA was performed, reducing the 14 dimensional dataset to a 7 dimensional matrix, though the last 4 factors have Eigenvalues that is smaller the 1, thus cannot be used. Looking at the Eigenvalues of the correlation matrix (table 23) we can see that the first value explains about 52% of the total variance, the second value about 20% and third approximately 2%. When considering the change in slope (graph 5), a distinct change is seen between the second and the third values, suggesting the use of the first two factors for the projection of the variables, which explains about 72% (71.9549%) of the total variance.

	Eigenvalue	% Total variance	Cumulative Eigenvalues	Cumulative %
1	7.215264	51.5376	7.21526	51.5376
2	2.858416	20.41726	10.07368	71.9549
3	2.06528	14.752	12.13896	86.7069
4	0.89038	6.35986	13.02934	93.0667
5	0.414117	2.95798	13.44346	96.0247
6	0.375285	2.68061	13.81874	98.7053
7	0.181258	1.2947	14	100

Table 23: Eigenvalues of correlation matrix, and related statistics for white wines, active variables only.

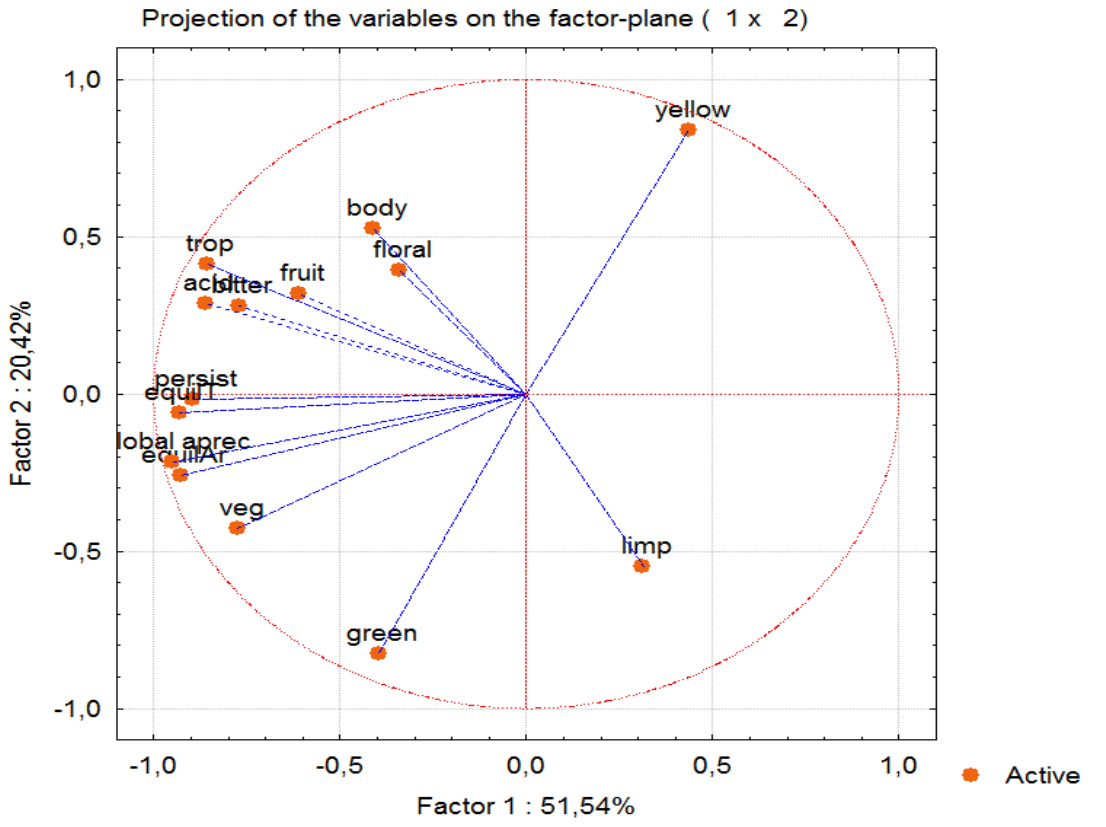


Graph 5: Eigenvalues of correlation matrix as a function of Eigenvalue number for white wines.

Table 24 presents the factor coordinates of the variables, based on correlation with the first and second factors chosen as the principal components for the sensorial analysis of the white wines. The values in each of the boxes represent a correlation coefficient. Those that are indicated by yellow color represent the most negatively correlated parameters, while the red ones represent the positive correlations. A projection of the variables on the factor plane was conducted, using these first and second factors/dimensions (graph 6).

		Factor 1	Factor 2
Color	Limpidity	0.313063	-0.54975
	Yellow	0.436105	0.839758
	Green	-0.396	-0.82498
Aroma	Fruity	-0.61159	0.320218
	Floral	-0.34325	0.393419
	Tropical notes	-0.85709	0.414559
	Vegetable	-0.77348	-0.42733
	EquilibriumAr	-0.92833	-0.25989
Taste	Body	-0.41157	0.527223
	Bitterness	-0.77254	0.280231
	Acid	-0.86258	0.289627
	Persistence	-0.89584	-0.01675
	EquilibriumT	-0.93275	-0.06082
	Global ap.	-0.94916	-0.21616

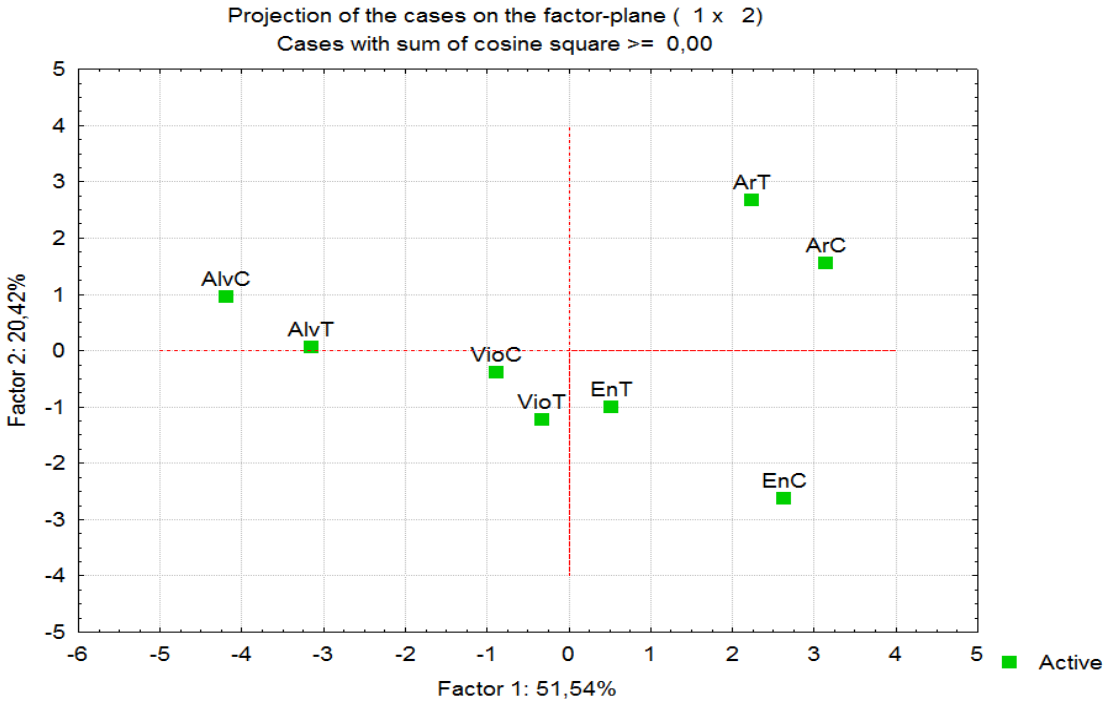
Table 24: Factor coordinates of the variables, based on correlations with factor one and two for the white wines.



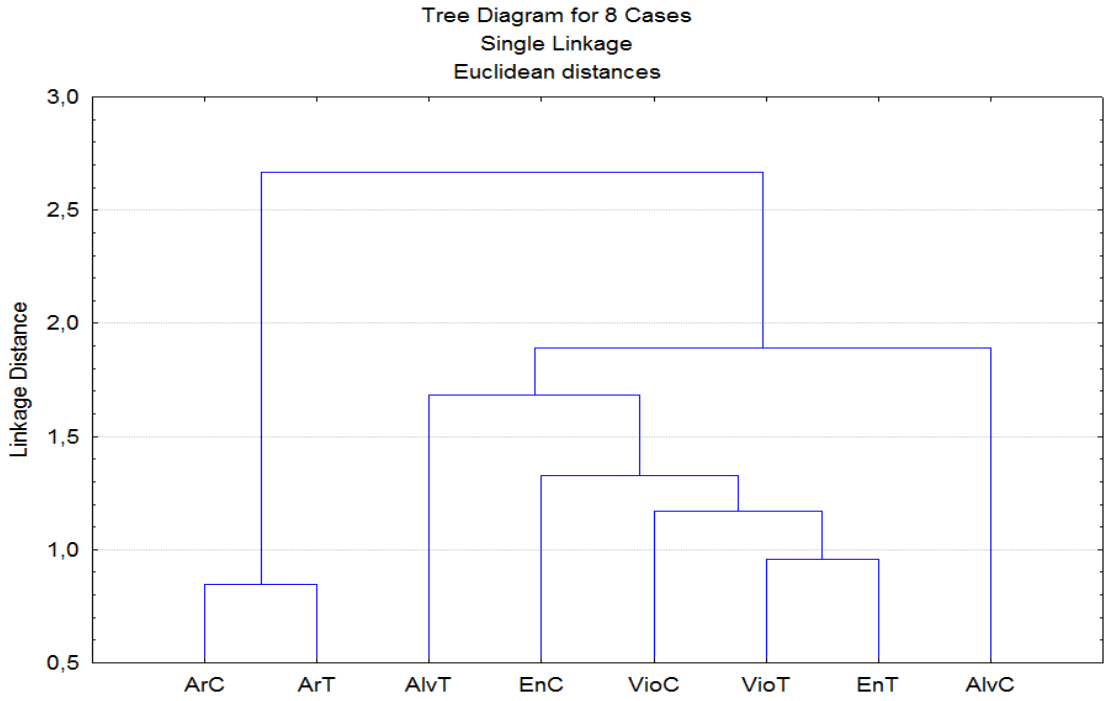
Graph 6: Projection of variables on the factor-plane, using Factor 1 and Factor 2, for the white wines.

Looking at the correlation results and the projection we can see that factor 1 (axe X) is negatively correlated with the majority of the taste parameters (bitterness, acid, persistence and equilibrium), and is also negatively correlated with tropical notes, the main parameter of interest in this research. Factor 2 (axe Y), is negatively correlated with the color green and positively correlated with the color yellow. When comparing these results with the projection of the wines (graph 7), it is seems that the Arinto wines (ArT and ArC) were characterized mostly by the color (factor 2), specifically by the parameter yellow. This is most likely due to the defect, rendering these wines as more oxidized, therefor less fitted for the aromas and tastes of this tasting sheet and more yellow as well, because of the oxidation defect. From this results it is seems that the wines presenting more of tropical notes, are also characterized with taste parameters, such as acid and persistence. The Alvarinho control (AlvC) wine is mostly characterized by these parameters and the Encruzado control (EnC) wine is the less one (graph 7). Additionally, we can see that different varieties are grouped together, suggesting again less impact of the treatment than that of the cultivator on the results.

A cluster analysis was conducted (graph 8) suggesting two groups; the first one is the Arinto wines, as expected, due to their defect. The second group is composed of the rest of the white wines with less differences between them. Both the PCA and the cluster analysis supports the ANOVA results, suggesting that the cultivator is more important in contributing to the differences in the sensorial perception than the current treatment and protocol used.



Graph 7: Projection of the different white wines (treatment and control), using Factor 1 and Factor.



Graph 8: Cluster analysis for the white wines.

5. Conclusions

The Objective of this study is to evaluate a new enological strategy to release volatile thiols from their precursors using a β-lyase enzyme in white and red wines. When considering the literature, this kind of an approach seems to be a new direction used to try and increase thiols concentration in wines. Utilizing the β-lyase enzyme as an additive may permit a wine-making style which is independent of the use of selected yeast for the release of thiols compounds.

The first study done in ISA (Chaves, 2012) evaluating this strategy was conducted with a main difference comparing to the present study; the use of skin contact (maceration) to promote extraction of thiols precursors located in the skin of the berry was added to this experiment.

In both studies, two red grape varieties were used (Syrah and Touriga Nacional) and four white grape varieties (Viosinho, Encruzado, Alvarinho and Arinto), all of each were checked for their chromatic profile and were evaluated by a sensorial analysis.

A general picture that can be illustrated from this study suggests no significant differences in the parameters (chromatic and sensorial) checked for both red and white varieties;

The chromatic parameters that did show a significant difference in both red and white wines could have been a result of the treatment and protocol used, which are designed to facilitate extraction of compounds from the skin of the grapes through the use of pecolytic enzyme and skin contact (such

as the increase of Tannin power for treated Syrah wines). Nevertheless, these differences did not manifest themselves during the wine tasting (either in scores or comments).

A sensorial analysis using PCA, ANOVA and Cluster Analysis was performed on the results of 10 experienced tasters, which supports that no significant differences between the treated and control wines (red and white) can be seen for the attributes checked. When considering the results for the red wines, a difficulty to differentiate between the control and treated wine was clearly presented through all of the statistical tools used, though it can also be concluded from the PCA that the Touriga Nacional variety is more characterized by floral notes and the Syrah by vegetable notes. The cluster analysis performed suggested that Touriga Nacional may be more responsive to the treatment than Syrah, which concords with Chaves, (2012) who found a greater increase in thiol compounds for Touriga Nacional. It may be concluded that any future study will preferably focus on this grape variety rather than Syrah.
In terms of the white wines, a similar situation is presented, where there is no statistical validation for any of the differences between the wines. Unlike the red wines, the cluster analysis did not provide any strong insights into which of the varieties reacted better to the treatment.

For both red and white wines, it is important to mention again the synergic or antagonistic effects among the volatile compounds in the wine's matrix that could enhance or depress each other (Roland *et al.*, 2011b), suggesting a possible increase in thiols, though not sufficient to be detectable in a sensorial trial as in this study.

Lastly, these varietal thilols are easily oxidized, and may react with compounds such as quinones, a reactive electrophilic oxidation intermediate in the wine. (Waterhouse *et al.,* 2012). The loss of varietal thiols due to the vinification conditions of the wine (being a mini-vinification) could present itself as the main reason for the observed wine tasting results. A mini-vinification such as this reults in a bigger surface area to volume ratio, thus more air contact and possible oxidation. Changing the scale of vinification and increasing the volume may result in less loss of varietal thiols and different sensorial impression.

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Annex

Table 25: The enological products used for the vinification of white and red wines.

Enological product	Objective of The product	White w.	Red w.
Aromax B4®	Must antioxidant containing sulfur dioxide and ascorbic acid, to facilitate the oxygen reduction in musts and wines. The action characteristic of this product is granted by the innovative immobilization system of the active principles – tannin, Vitamin C and pyrosulphite – on inert floating agents.	**+**	**-**
Endozyme ICS 10 Arôme®	Pectolitic enzyme with specific activities that increase the transition of varietal aromas from the skin to the must and releasing terpene aromas. Polygalacturonose function related to the secondary cellulosic and hemi cellulosic activities.	+	-
Endozyme Muscat®	A specific pectolitic enzyme with a high pectinlyosic activity, integrated with secondary cellulosic activities, such as rhamnosidose and arabinose.	+	-
Enovit®	Provides suitable concentration of ammonia nitrogen, vitamins and micro-elements supporting the metabolic activity of the yeasts, which activates and regulates the fermentation.	+	+
Zymasil Bayanus®	Selected active dry yeast.	+	+
Fermoplus Energy Glu®	Energy booster for fermentations, contain amino-acids and vitamins.	+	+
Fermoplus Integrateur®	Vitamins, sterols, mineral salts and nitrogenous substances are in a molecular form readily assimilable by the yeasts.	+	+

Table 26: The full triplicate data for the chromatic parameters of the white wines.

	White Wines							
	Viosinho		Alvarinho		Encruzado		Arinto	
	Control	Endozyme Thiol	Control	Endozyme Thiol	Control	Endozyme Thiol	Control	Endozyme Thiol
Color Intensity (a.u)	0.146	0.136	0.163	0.159	0.087	0.093	0.245	0.232
	0.149	0.133	0.195	0.153	0.086	0.092	0.227	0.226
	0.147	0.147	0.218	0.160	0.086	0.098	0.228	0.225
Total Phenols (a.v)	9.95	10.38	11.14	11.43	9.99	10.30	10.72	10.82
	9.84	10.45	11.20	11.48	9.78	10.30	10.68	10.97
	10.09	10.23	11.03	11.37	9.89	10.41	10.72	10.92
Total Phenols (mg/L Gallic acid)	322.6	336.5	361.1	370.4	323.8	333.9	347.5	350.7
	319.0	338.7	363.0	372.1	317.1	333.9	346.2	355.6
	327.1	331.6	357.5	368.5	320.6	337.4	347.5	353.9
Total Flavo. (mg/L Catechin)	121.6	135.8	135.2	136.1	140.7	116.7	127.4	124.5
	125.5	127.4	133.2	136.8	227.7	121.6	123.2	123.2
	114.5	135.8	135.8	133.6	111.9	121.3	127.4	124.5
Total Non Flavo. (mg/L Gallic acid)	496.8	496	558.4	579.2	452.8	536.8	544	559.2
	478.4	523.2	568	581.6	220.8	524.8	551.2	574.4
	525.6	484	548	580.8	516	534.4	544	567.2

Table 27: The full triplicate data for the chromatic parameters of the red wines.

	Color Intensity (a.u)		color Shade (a.u)		Total Pigments (a.u)		Poly.Pigment (a.u)	
	Control	Endozyme Thiol Rouge	Control	Endozyme Thiol Rouge	Control	Endozyme Thiol Rouge	Control	Endozyme Thiol Rouge
Syrah	9.06	8.70	0.690	0.690	24.75	25.45	2.71	2.65
	9.05	8.57	0.688	0.700	26.77	27.98	2.60	2.63
	9.00	8.74	0.691	0.688	26.06	25.76	2.66	2.68
Touriga Nacional	16.96	17.17	0.767	0.738	35.05	34.04	4.75	4.83
	16.26	17.66	0.762	0.726	36.06	35.65	4.90	5.01
	16.28	17.04	0.764	0.745	36.16	33.33	4.90	5.00
	Total Antho. (mg/L)		Colored Antho. (mg/L)		Ionization Index (%)		Poly. Index (%)	
Syrah	404.6	420.7	38.0	36.0	9.4	8.6	11.0	10.4
	448.6	471.9	40.2	34.6	9.0	7.3	9.7	9.4
	432.5	425.8	38.2	36.0	8.8	8.5	10.2	10.4
Touriga Nacional	542.6	519.7	66.6	70.4	12.3	13.5	13.6	14.2
	557.8	546.1	58.0	71.6	10.4	13.1	13.6	14.1
	559.8	499.9	57.8	64.8	10.3	13.0	13.6	15.0
	Total Phenols (a.u)		Total Phenols (mg/l Gallic acid)		Total Non Flavo. (mg/L Gallic acid)		Total Flavo. (mg/L Catechin)	
Syrah	42.800	42.800	1385.7	1385.7	177.9	173.0	2985.6	2997.6
	43.200	42.800	1398.6	1385.7	181.8	170.8	3008.0	3003.2
	42.500	43.700	1376.0	1414.8	175.6	173.4	2967.2	3068.8
Touriga Nacional	74.100	73.100	2398.6	2366.2	208.6	203.1	5413.6	5347.2
	75.700	74.400	2450.4	2408.3	2268.2	215.1	5490.4	5469.6
	75.700	73.600	2450.4	2382.4	216.2	223.8	5525.6	5400.8
	Tannin Power NTU/ml)							
Syrah	142.000	156.750						
	138.875	158.250						
	149.750	159.125						
Touriga Nacional	247.125	272.125						
	290.750	284.125						
	325.625	271.875						